CYTOMEGALOVIRUS INFECTION OF DENDRITIC CELLS:

Role of Immune Cell Tropism in the Viral Life Cycle

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A Dissertation in Molecular Microbiology and Immunology

Presented to the faculty of Oregon Health & Science University in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

September 2008

School of Medicine

Oregon Health & Science University

CERTIFICATE OF APPROVAL

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TABLE OF CONTENTS

Acknowledgements

The work presented in this dissertation is due in large part to the collective efforts, encouragement and support of so many people, too numerous to name in just a few pages. I would first like to acknowledge my mentor, Dr. Jay Nelson. His contributions to the field of Herpesviruses are well recognized both at home and abroad, and are an inspiration to this scientist. Dr. Nelson is the consummate "devil's advocate", and has gone to great pains to ensure that the most challenging questions his students face come directly from him and as such has prepared his students for critical review within the broader scientific community. Working in his lab was both a challenge and a privilege and I thank him for the opportunity. Likewise, I owe a great deal of gratitude to Dr. Daniel Streblow for his patience, expertise and his counsel. In addition to guiding this project, Dr. Streblow has provided a wonderful example in the art of balancing science and family life, for that and so much more I am thankful.

While I knew when I set up my thesis committee that I had grouped together the leading experts in the field, it was not until I was knee deep in writing that I realized to what extent each member of my committee had contributed to the field as a whole. Drs. William Britt, David Johnson and Klaus Früh have all contributed greatly to this work as well as my development as a scientist. The members of my *dream team* committee have all gone above and beyond in terms of their guidance. For their time, support, and insistence on *more*, I am forever grateful.

I would also like to extend my sincerest gratitude to one teacher in particular who encouraged and prepared me for this journey. Sr. Barbara Mary Pharis , introduced me to the wonders of science including my first introduction to evolution and my first experiment; she lit a fire within an eight year old's heart to better understand nature and the mysteries that God has laid out before us. In addition to her encouragement for my own development, Sr. Barbara always highlighted my responsibility to the greater community; that I will not forget. Nearly 25 years of her encouragement has made this day in my life possible.

My husband, Dr. John M. Jones, who rescued me from the quick sand that was Organic Chemistry, has been the calm in this storm, my own personal motivational speaker, my laugh mate, my very dear friend, the cherry on the top of my sundae. Quite a team we have become on this road. This would not have been possible with out him. John has been my sounding board, my confidant, my therapist, and has made it all worthwhile. Along the way we added one more to our clan, Maya Lucia, my light, my love, my angel. She has put all things into perspective and shed a brighter light on each day.

Finally, I am forever indebted to my parents, Patricio and Marcia Baca, first for always prioritizing my education over ALL else. My parents always put my education first at the cost of everything else in their own lives and as such are a beautiful example of selfless parenting. What other families saw as necessities

my parents went without, so that I might have access to the best education available. For believing in me, and not in the "you can do anything you want to do" cliché way that parents so often do, but in the Baca way: "You can do anything you get off your tush and make happen" way...for never letting a B+ slide by without asking why not an A...for never letting me feel entitled to anything, except respect that is earned...for instilling the idea that if you want something strongly enough, you should work harder than anyone to attain that goal...for always reminding me that my thoughts are tangible entities and become real in one form or another...I will never forget that this achievement is a generational effort, won on the backs of the people whose love and commitment made it happen one sugar beet, one onion, one letter, one bullet at a time.

ABSTRACT

Human Cytomegalovirus (HCMV) dedicates a major portion of its genomic resources to both evasion and modulation of the host immune response, resulting in a relatively benign life-long association with the healthy host. Infection of antigen presenting cells, including dendritic cells and macrophages, provides a unique opportunity for the virus to simultaneously evade and manipulate the host immune response by hijacking a cornerstone in the network of immune mediators. While both Mouse CMV and Human CMV have been demonstrated to infect dendritic cells and down regulate MHC II, infection efficiencies and the limited depletion of MHC II in the current *in vitro* models have hampered biochemical analysis of MHC II depletion in the context of DC infection. Infection of bone marrow derived dendritic cells with a recombinant Rat CMV expressing GFP under the cellular promoter, eF1α, facilitated the tracking of infected cells by flow cytometry and fluorescence microscopy, further delineating the effects of direct infection versus soluble factors mediating MHC II depletion. Furthermore, due to the high rate of infection and near complete loss of MHC II in infected DCs, biochemical analysis including western blot of steady state MHC II, immunoprecipitation and pulse chase metabolic labeling were utilized to explore mechanisms of RCMV depletion of MHC II. The body of work detailed here documents the development of the RCMV BMDC infection model and a novel mechanism (lysosomal mediated degradation) by which Rat Cytomegalovirus down regulates MHC II expression in bone marrow derived dendritic cells.

Furthermore, the development of two additional model systems to dissect the roles of CMV tropism for specific cell types in the viral life cycle and the requirements for the establishment of persistence are described.

CHAPTER ONE: INTRODUCTION

A. CYTOMEGALOVIRUS

1. CLINICAL OUTCOMES OF CMV DISEASE

Recent studies estimate that nearly 136 million individuals, 58.9% of the total population, in the United States alone, are infected with Cytomegalovirus. Infection statistics estimate that between 60-90% of the world's adult population are seropositive. Although infection with the virus typically results in a subclinical primary infection of immunocompetent individuals, HCMV is a common opportunistic pathogen of the immunocompromised. Those particularly susceptible to complications arising from infection include transplant recipients, neonates, cancer patients, HIV positive individuals and patients undergoing immunosuppressive therapy. Given the prevalence of CMV in the general population coupled with an increasing number of individuals undergoing allograft transplantation and immunosuppressive therapy, CMV is an ever-present public health concern.

a. Infection in the Immunocompetent Host

Viral replication within the mucosal epithelium is believed to initiate a natural CMV infection within the immunocompetent host upon direct exposure to infectious secretions such as saliva, urine, and genital fluids. Following the initial replication

at the primary site of infection, tissues in the periphery are seeded. The subsequent systemic infection is presumed to be dependent on a cell associated viremia, as cell free virus is not detected in the blood (1, 169, 172). The current model for the dissemination of CMV within the host depends upon cells of the myeloid lineage, monocytes in particular, trafficking to the site of initial replication, where they are in turn infected. While monocytes are susceptible to infection with CMV, they are not permissive for the full replication cycle of the virus until they have terminally differentiated into macrophages. It is presumed that the terminal differentiation and viral replication that ensues leads to the spread of CMV in the periphery. Once seeded, the salivary gland and kidney tubule ductal epithelia remain sites of persistent replication and release into the saliva and urine respectively, thus providing a vehicle for viral spread between hosts. During the systemic phase of primary infection, the greatest levels of persistent viral shedding can be found in the urine, saliva, genital secretions and breast milk. The onset of an effective cell-mediated immune response correlates with clearance of acute infection in immunocompetent individuals. Although the host mounts an impressive immune response, clearance of the virus is not complete.

b. Infection in the Immunocompromised Host

Infection in the immunocompromised host, much like in the immunocompetent host, can be the result of a primary infection, a reactivation of a latent infection or a reinfection. The primary difference in the disease course of individuals with

compromised immune systems compared to the otherwise healthy host is the loss of immune control of viral replication and the subsequent rapid dissemination. Several studies have linked the severity of disease caused by acute infection of immune compromised patients, such as AIDS patients and transplant recipients, with a rapid onset of elevated viral titers in the blood (57, 58). This is believed to be reflective of uncontrolled virus replication due to a lack of immune control and a predictor of widespread dissemination in the peripheral organs (57, 58). The most severe cases of CMV disease are found in allogeneic stem cell recipients and late stage AIDS patients (175).

c. Disease Associated with Acute Infection

Acute infection of the immunocompetent host is typically asymptomatic, however it can result in a mononucleosis like illness complete with reports of general malaise, soar throat, splenomegaly and a fever lasting longer than ten days (120, 127, 191). On average, patients were symptomatic for nearly eight weeks. Several reports have estimated the occurrence of even minor symptoms associated with CMV infection only occur in 5-10% of primary infections among immunocompetent individuals (83, 196, 260). Rarely, more acute symptoms are associated with infection of immunocompetent individuals. These can include enteritis, retinitis, hemolytic anemia, thrombocytopenia, mycocarditis, ocular disease and neuropathies.

Symptomatic congenital infection can be characterized by microcephaly, seizures, "poor feeding", jaundice, low birth weight, abnormal cranial CT scans and hearing loss (21). Among congenitally infected newborns, only 5-10% are symptomatic. However, current reports estimate that 50-90% of those symptomatic newborns will be impaired over their lifetime with some combination of CMV related disabilities including: mental retardation, auditory defects, cerebral palsy and vision loss; approximately 10% of symptomatic neonates succumb to CMV disease.

d. Disease Associated with Chronic Infection

Chronic HCMV infection has been associated with a number of diseases, including chronic inflammatory diseases and vasculopathies such as atherosclerosis, transplant vascular sclerosis and restinosis (reviewed in (24). HCMV has been linked in the literature to a number of different pathologies, including more recently glioblastoma, but until a specific etiology can be detailed these observations are best approached cautiously. That said, there is an abundance of data in the literature to suggest that CMV does indeed accelerate the progression of chronic graft rejection, atherosclerosis and restenosis (85, 160).

One indicator that CMV is involved in the acceleration of graft rejection are the findings of multiple studies where treatment of patients with ganciclovir resulted in

a reduction in the rate of graft failure as compared to control groups (107, 163, 285). *In situ* hybridization revealed the presence of CMV DNA in the coronary arteries of allografted hearts with severe arteriosclerosis, whereas samples taken from control heart allograft patients without sign of vasculopathy were CMV negative (108, 309). HCMV has been shown to infect a multitude of cell types involved in the development of transplant vascular sclerosis (TVS) including smooth muscle cells (SMC), macrophages (Macs), endothelial cells (ECs) and fibroblasts. Several labs have followed up on the finding of CMV+ cells in the TVS lesion with *in vitro* studies to elucidate possible mechanisms of CMV induced TVS. Streblow and colleagues found that the HCMV encoded chemokine receptor, US28, is able to mediate SMC migration (267). Multiple studies, both *in vitro* and *in vivo* have also shown that CMV infection of endothelial cells leads to the upregulation of specific glycoproteins facilitating the adhesion of leukocytes to the infected cells (250-252). Use of rat transplant models has further supported a role for CMV induced graft rejection and shed light on several likely mechanisms. For example the dependence of CMV induced graft rejection on the recipient alloreactive immune response was made clear with the use of both a rat cardiac transplant and bone marrow chimera model where induction of donor tolerance in the recipient rat prevented CMV accelerated chronic rejection (189). Although the precise mechanism(s) through which CMV mediates graft rejection are still being delineated, there appear to be multiple roles for CMV involvement.

e. Antivirals

Anti-CMV pharmaceuticals and their mechanism of action

Currently five pharmaceuticals are approved for the treatment of acute systemic HCMV infection in immunocompromised patients and prophylactic or preemptive treatment of CMV disease in transplant recipients. While each of the approved five have been shown to significantly reduce or eliminate the viral burden and concomitant disease associated with CMV infection, their use has been restricted to treatment of the immunocompromised due to the potential toxicity associated with each of the drugs.

Four of the five approved antiviral drugs inhibit replication of HCMV by targeting the viral DNA polymerase (reviewed in (165). Cidofovir, Aciclovir, Ganciclovir, and its orally bioavailable valine ester, Valganciclovir, in their triphosphorylated form, are nucleotide analogs that act to either slow the viral DNA polymerase or terminate chain elongation altogether. Foscavir is a pyrophosphate analog which inhibits the viral DNA polymerase by selectively binding the pyrophosphate binding site on the viral polymerase at concentrations which do not affect the cellular polymerase. The fifth antiviral drug, Fomivirsen, is an antisense oligonucleotide complementary to the mRNA of the major immediate early 2 gene of CMV; it exerts its anti-CMV activity by inhibiting the translation of the IE2 mRNA.

Development of drug resistance

While mutations conferring CMV resistance can be found in the UL54 ORF encoding the viral polymerase, the molecular basis for the vast majority of drug resistant HCMV isolates can be traced back to mutations in the UL97 gene (reviewed in (59). The protein encoded by UL97 shares some homology with known protein kinases and bacterial phosphotransferases and has been demonstrated to phosphorylate Ganciclovir and Aciclovir to their monophosphate form *in vitro*. Once monophosphorylated, the drugs can then be further phosphorylated to the "active" triphosphate form (which inhibits the viral polymerase) by cellular kinases. Cidofovir is manufactured in the monophosphorylated form and as such does not require phosphorylation by the UL97 kinase. Likewise the pyrophosphate analog, Foscavir, and the anti-sense oligonucleotide, Fomivirsen, do not require phosphorylation by UL97 to exert their anti-viral function, thus making them resistant to selective mutations in UL97. The development of Foscarnet resistance has been attributed to mutations in the UL54 ORF encoding the viral polymerase (12). More recently a fomivirsen resistant mutant was isolated and interestingly, was shown to still contain the IE2 target sequence intact (178).

2. HISTORICAL OVERVIEW

The characteristic "owl's eye" (large inclusion bearing infected cell) morphology was first described in 1881 by H. Ribbert (209) [FIGURE 1]. His initial

observation described enlarged infected cells containing intranuclear inclusions surrounded by a clear ring. Around the time Ribbert published his 1881 findings, similar reports were published describing the same phenomena found in kidney, lung and liver tissues obtained from a fetus reportedly infected with Syphilis (114, 209, 217). Although as early as 1921 a viral agent was believed to be the cause of the "cytomegalia", it was not until 1956 that live virus was isolated by Smith, Rowe *et. al.* and Weller *et al.* each working independently (225, 244, 245, 300). Shortly after isolation of the virus, Weller and colleagues proposed a change in terminology from Cytomegalic Inclusion Disease (CID)-salivary gland virus to the currently accepted moniker, Cytomegalovirus (299). Since the initial isolation of CMV and subsequent advancements in serological techniques and *in vitro* viral culture, CMV is recognized as a significant pathogen of both the fetus and neonate. Congenital infection with CMV can lead to major sequela in the fetus and neonate including microcephaly, intracranial calcifications, mental retardation and auditory defects (114, 169). In fact, CMV is currently recognized as the leading infectious disease cause of brain damage in U.S. children. Clinical significance of CMV infection was further realized with the advent of the HIV/AIDS epidemic. Before the use of Highly Active Antiretroviral Therapy (HAART), CMV was the most common opportunistic viral pathogen afflicting AIDS patients, often presenting as chorioretinitis, a retinal infection.

The next 25 years yielded much advancement in our understanding of CMV molecular virology, including a greater understanding of the viral replication

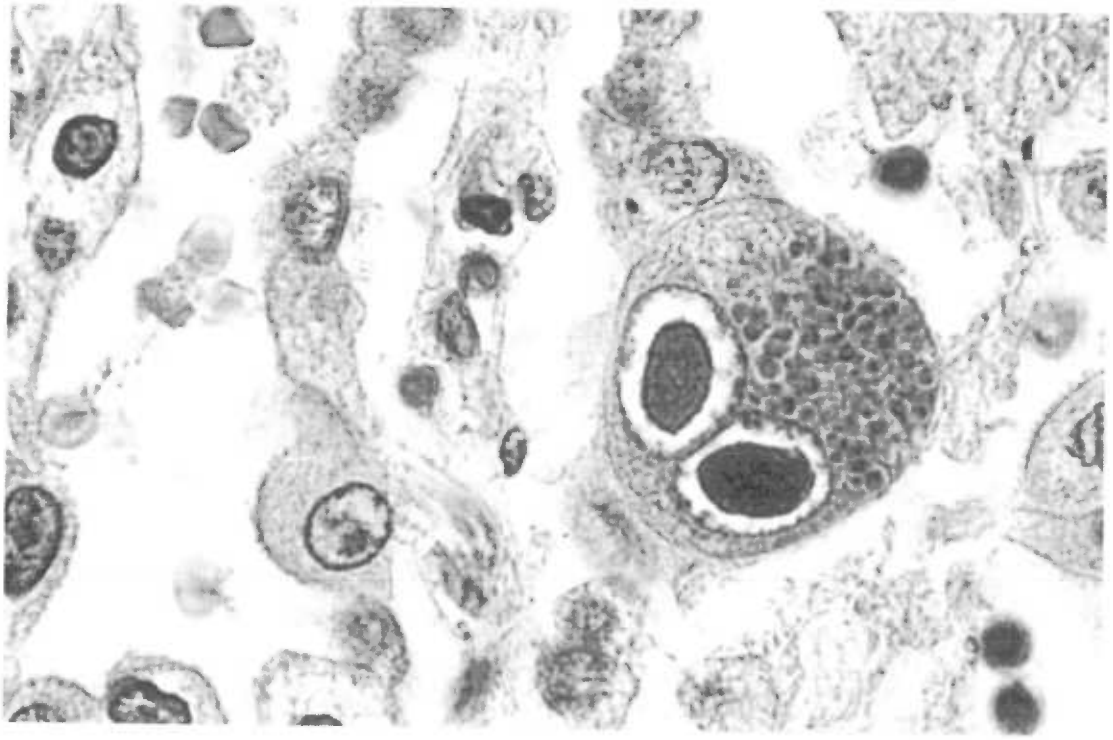

Figure 1. CMV OWL'S-EYE INCLUSION

H&E stained lung sample displaying CMV owl-eye inclusions

Image by Dan Wiedbrauk, Ph.D., Warde Medical Laboratory

Ann Arbor, MI

www.asm.org/Division/c/viruses.htm

cycle, genetic makeup, and interaction with the host immune system. Genetic analysis of CMV was accelerated by several key milestones. The laboratory strain of HCMV, AD169, was sequenced in 1990, followed by the sequencing of MCMV Smith strain and RCMV Maastricht strain in 1996 and 2000. CMV reverse genetics was then made possible and subsequently more efficient by the development of cosmid cloning systems and Bacterial Artificial Chromosome (BAC) clones containing the entire viral genome.

3. MOLECULAR REVIEW

a. Classification

The *Herpesviridae* family includes more than 120 different viruses infecting primarily vertebrate hosts. This diverse collection of viruses was historically grouped based on virion structure. A prototypical herpesvirus consists of a linear double stranded DNA genome enclosed in an icosahedral capsid approximately 125nm in diameter. The capsid, surrounded by an amorphous protein layer, termed the tegument, is engulfed in an envelope studded with viral glycoproteins. This generalized classification scheme based on viral architecture has lead to the inclusion of a diverse group of viruses within the herpesvirus family, including those that infect mammals, reptiles, amphibians and even bivalves.

Division of the *Herpesviridae* family into three subfamilies, *Alphaherpesvirinae, Betaherpesvirinae* and *Gammaherpesvirinae* is based upon gross biological properties including host range, cellular tropism and growth characteristics (219). Members of the *Alphaherpesvirinae* subfamily are grouped based on their variable host range, neurotropism and rapid replication cycle. Viruses within this subfamily induce significant cytopathic effect on the infected host cell and are able to maintain a latent infection in sensory ganglia. The common traits shared by members of the *Betaherpesvirinae* subfamily include a narrow host range, widespread tissue dissemination, long reproduction cycle, and enlargement of infected cells (cytomegalia). Additionally, viruses belonging to the *Betaherpesvirinae* subfamily establish a latent or low level persistent infection in a variety of tissues. The subfamily *Gammaherpesvirinae* is composed of lymphotropic viruses (usually specific for either B or T cells) with a restricted host range. Members of the *Gammaherpesvirinae* rarely induce a lytic infection, but rather maintain a latent infection in lymphocytes or endothelial cells. Disease associated with members of the *Gammaherpesvirinae*, is more commonly due to the transformation of infected cells, leading to abnormal growth such as lymphoproliferative disorders, rather than direct cytopathic effect of viral infection.

Of the more than 120 herpesviruses that have been documented to date, only eight Human Herpesviruses (HHV) have been identified: HHV 1 (Herpes Simplex Virus 1), HHV2 (Herpes Simplex Virus 2), HHV3 (Varicella zoster), HHV4 (Epstein-Barr Virus), HHV5 (Cytomegalovirus), HHV6 (Roseolovirus),

HHV7 (Roseolovirus), HHV8 (Kaposi's Sarcoma Associated Herpesvirus). Varicella zoster, Herpes Simplex Virus 1 and 2 are all members of the *Alphaherpesvirinae*. The Roseolovirus and Human Cytomegalovirus (HCMV) are members of the subfamily *Betaherpesvirinae.* Epstein-Barr and Kaposi's Sarcoma Associated Herpesvirus are members of the *Gammaherpesvirinae* subfamily.

b. Structure

The HCMV virion measures 200-300nm in diameter with a somewhat pleiomorphic envelope (291). Encased within the envelope is the 125nm capsid, coated in a proteinaceous layer termed the tegument, primarily consisting of four viral encoded proteins: pp65 (UL83 gene product), pp71 (UL82 gene product), pp150 (UL32 gene product) and largest tegument protein (UL48 gene product) (291). The icosahedral capsid is composed of five core proteins common to all herpesviruses: major capsid protein (MCP, the UL89 gene product), minor capsid protein (TRI1, the UL46 gene product), minor capsid binding protein (TRI2, UL86 gene product), the smallest capsid protein (SCP, the UL48A gene product) and the portal protein (PORT, the UL104 gene product) (26, 77, 173, 174).

Encased within the capsid is the viral genome, comprised of a double stranded linear DNA molecule ranging in size from 196-241 kbp in length (49, 52). Conservative estimates predict a coding capacity of 166 open reading frames,

whereas more liberal predictions estimate a coding capacity in excess of 200 ORFs (49, 52). Similar to members of the alpha herpesviruses, the HCMV genome is composed of two unique sequence segments (U_L and U_S), each bracketed by inverted repeat sequences termed internal repeat long (IRL), terminal repeat long (TRL), internal repeat short (IRS) and terminal repeat short (TRS). These direct terminal repeats contain cis-acting elements mediating the cleavage and packaging of nascent viral genomes into four distinct isomers. RhCMV, MCMV and RCMV genomes do not isomerize (175).

c. HCMV Replication Cycle

The initial steps in the CMV replication cycle are a two-step process composed of adsorption (or binding) of the virion to the cell surface and penetration of the plasma membrane. Binding of the virion appears to involve interaction with a number of charged molecules, such as heparan sulfate and chondroitin sulfate glycosaminoglycans (GAGs), on the surface of the cell (238, 254). The viral glycoproteins required for penetration (in the case of HSV) are not required for adsorption to the cell surface (117).

Penetration of the virion into the cytoplasm involves the fusion of the viral envelope with the host cell plasma membrane. Interactions between viral glycoproteins and cell surface receptors trigger the fusion of the virion envelope and the plasma membrane (42, 64, 297, 298). Additionally, a recent report

suggests that HCMV may employ an alternate method to enter the cell. Studies performed by Ryckman *et al.* have demonstrated that HCMV entry into endothelial and epithelial cells occurs by endocytosis and low pH-dependent fusion (226). HCMV entry into fibroblasts is dependent upon the interaction between viral glycoproteins, gC and gB, and fibroblast cell surface receptors, such as EGFR, integrins and GAGs; this interaction triggers the fusion of the virion envelope and the plasma membrane (42, 64, 297, 298). While the full complement of both viral glycoproteins and host cell surface receptors that mediate *HCMV* entry into the variety of permissive cell types have yet to be defined, more is understood about the complex nature of *HSV* entry. Four HSV glycoproteins have been demonstrated to be essential to fusion, gB, gD, gH, gL (34, 72, 142, 223, 254).

Once the viral envelope has fused with a cellular lipid bilayer, nucleocapsids are actively transported via the microtubule network to the nuclear pores where the viral genome is deposited (186). The host transcriptional machinery, including RNA polymerase II is utilized to synthesize viral mRNA. HCMV transcription in fibroblasts cultured *in vitro* proceeds in a temporal cascade where three classes of genes are transcribed in succession: immediate early (IE), early (E) and late (L), also known as α, β and γ.

Pharmacological inhibitors of viral DNA replication and protein synthesis have been historically used to define the three classes of Herpesvirus genes in

cultured cells. While the classification of CMV genes into three kinetic classes has been a useful tool for understanding CMV replication in cultured fibroblasts, recent analyses of global transcription patterns *in vivo*, as well as *in vitro* cultured cells suggests both a cell type and tissue specific CMV transcriptional profile *in vivo* (269).

Transcription of the IE class of genes occurs "immediately" after the deposition of viral DNA into the nucleus. Treatment of cells with protein synthesis inhibitors, such as cyclohexamide, blocks the synthesis of early and late genes, but does not affect the transcription of the IE genes, thus pointing to the dependence of E and L transcription on *de novo* protein synthesis. The proteins encoded by the IE genes are typically transcription factors required for the continued transcription of the subsequent kinetic classes of viral genes as well as immune evasion mediators. Transcription of the early genes is dependent upon the action of the IE gene products and as such can be blocked by inhibitors of protein synthesis. Genes are further defined as belonging to the Early class if found resistant to DNA synthesis inhibitors, such as Foscarnet and Ganciclovir. The Early genes comprising the second class of CMV genes to be transcribed in fibroblast culture include genes involved in the replication of the viral genome.

The Late genes are transcribed after replication of the viral genome and include virion structural components necessary for the generation of new virions. Due to their dependence upon both the Immediate Early and Early classes of genes,

transcription of L genes is sensitive to both inhibitors of protein and viral DNA synthesis.

Once viral proteins are synthesized, capsid structural components are translocated to the nucleus where they assemble into a protein scaffold that ultimately forms the capsid. Replication of the viral genome begins at the OriLyt and proceeds to form long concatemeric DNA via the rolling-circle method of DNA amplification. Viral genome is packaged into the nascent capsids; cleavage at conserved sites within the genome ensure a unit length or "head-full" packaging of one complete viral genome per capsid. Once the viral genome is packaged into the capsid the newly formed nucleocapsid exits the nucleus. Herpesvirus egress has been a controversial topic of research, with multiple theories as to where the virus acquires an envelope (164). More recently, a model of envelopment/de-envelopment has gained wider acceptance (164). Within this model, the nucleocapsid is believed to aquire an envelope as it buds through inner nuclear membrane. This envelope is lost as it fuses with the outer nuclear membrane, thus releasing naked capsid into the cytoplasm, where tegumentation is believed to occur. The tegument coated capsid then acquires its final envelope when it buds into the Golgi. Finally, release of viral progeny occurs through the lysis of the infected cell or by budding of the virus through the plasma membrane.

Figure 2.

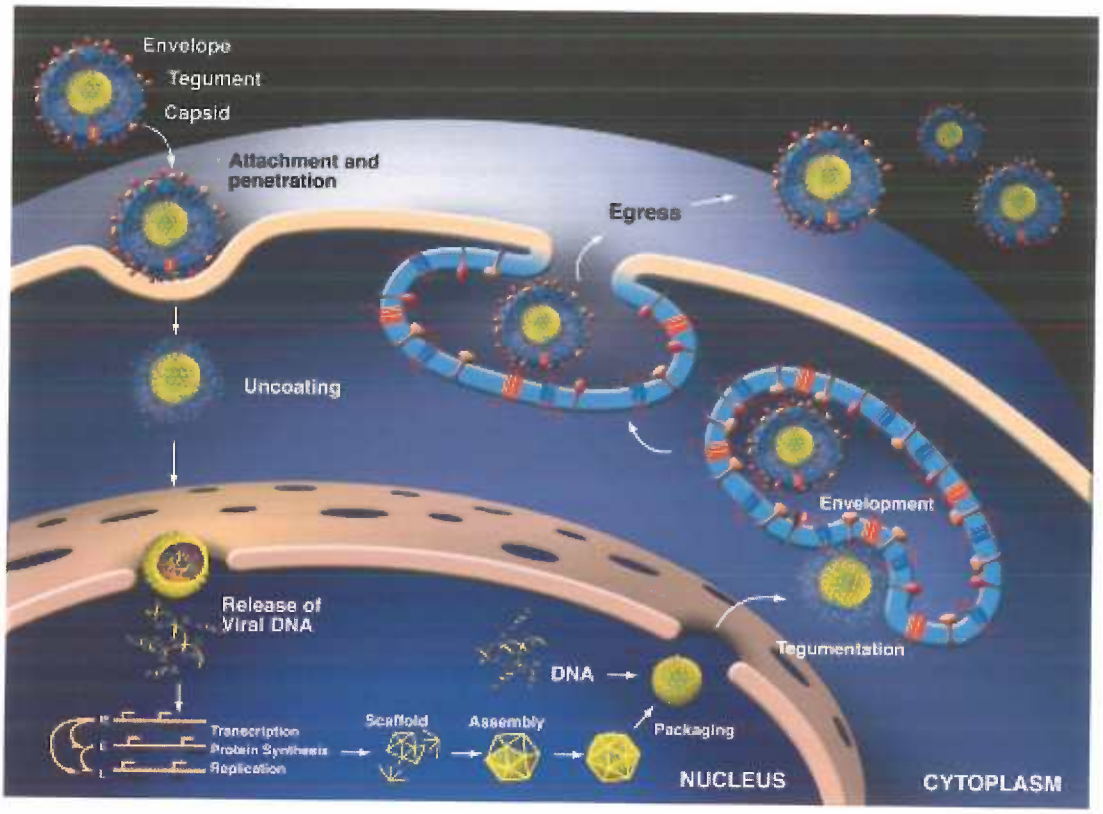
Envelope
Tegument
Capsid
Attachment and penetration
Egress
Uncoating
Envelopment
Release of Viral DNA
Tegumentation
DNA
Transcription
Protein Synthesis
Replication
Scaffold
Assembly
Packaging
NUCLEUS
CYTOPLASM

Figure 2. CMV SINGLE CELL REPLICATION CYCLE

The initial events of the HCMV replication cycle involve virion binding to the surface of the cell, attachment and penetration. Binding of the virion to cell surface receptors (as yet not fully defined) triggers fusion of the viral and plasma membranes, resulting in the uncoating of the virion and deposit of the nucleocapsid into the cytoplasm. The viral nucleocapsid transits to the nucleus where the viral genome is released. Transcription in vitro occurs in a cascade of three kinetic classes of genes: Immediate early, Early, and Late. Viral progeny are generated upon DNA synthesis and transcription of Late genes encoding virion structural components. Assembly of the capsid is followed by packaging of the viral genome and egress out of the nucleus. Envelopment is believed to occur as the virus transits through the trans golgi network and subsequently buds through TGN membranes. Mature enveloped virions are packaged in vesicles which subsequently fuse with the plasma membrane.

3. CMV ANIMAL MODELS

Due to the strict species specificity of CMVs for their natural host, animal models of CMV infection are critical to the understanding of infection *in vivo*. Since Human Cytomegalovirus is unable to productively infect other species, the study of CMV infection *in vivo* has been largely restricted to examination of infection of various species with their cognate CMV. Several animal models have emerged over the years and each has provided a unique paradigm within which various aspects of CMV biology have been explored.

The murine model of CMV infection is by far the most utilized of the CMV animal models. Small animal models in general facilitate the establishment of statistical relevance at a minimal cost (relative to the larger animal model systems). An even greater benefit of the murine model is the ability to genetically manipulate the host, such that specific host factors can be tested for their contribution to CMV biology. The plethora of readily available transgenic mice combined with the vast library of antibodies and recombinant mouse cytokines have greatly advanced our understanding of the immune response to CMV. In particular, studies utilizing the MCMV model have identified a hierarchy of protective immune responses to CMV infection. Use of knock out mice with specific deficits in NK cells, B cells, T cells have facilitated our understanding of the immune response to CMV, and the critical role each cell type plays in moderating CMVpathogenesis at various stages of infection. In addition, the MCMV

infection of mice provides an *in vivo* model to study persistence, latency and dissemination.

Advancements made in microvascular surgery in combination with the rat model have greatly expanded our understanding of CMV acceleration of vasculopathies and associated graft rejection (reviewed in (121). Deciphering the underlying mechanisms of Human CMV accelerated transplant vascular sclerosis (TVS) has proven to be quite a challenge for a multitude of reasons (reviewed in (265). One, the high degree of prevalence of HCMV infection within the population makes identification of HCMV seronegative cohorts extremely hard to come by. Two, defining a relationship between the timing of infection and the onset of disease associated is nearly impossible in human subjects. Three, TVS is a complicated process and the major players in TVS development, inflammation, endothelial cells, macrophages, fibroblasts and smooth muscle cells all play roles in the lifecycle of CMV *in vivo*. For the afore mentioned reasons, the Rat CMV transplant model has proven quite valuable in elucidating the underlying mechanisms of CMV induced TVS.

The guinea pig model has likewise provided insight into specific aspects of CMV pathogenesis (reviewed in (231). Unlike Mouse CMV, Guinea Pig CMV crosses the placental barrier, leading to fetal infection, providing a small animal model for the study of congenital infection (231). Congenitally infected guinea pigs present with a variety of sequelae similar to human neonates including CNS involvement

and inner ear damage that can result in deafness (82, 306). Because GPCMV crosses the placenta, the model is a useful tool in the study of maternal correlates of protective immunity and preconceptual vaccination strategies (229, 230). Future studies will likely be aided by the recent development of the GPCMV Bacterial Artificial Chromosome.

While the rodent CMVs provide both cost effective and easily manipulated models for the *in vivo* study of CMV pathogenesis, the degree of genetic divergence between Human CMV and the small animal model CMVs prevents direct correlation of findings in MCMV, RCMV and GPCMV back to Human CMV (reviewed in (25). Chimpanzee CMV is the most closely related non-human animal model of HCMV, however chimpanzees are a protected species and as such biomedical research in chimps is highly prohibitive (49). The Rhesus macaque non-human primate model has been widely utilized in biomedical research, whereas other primates are of limited use due to ethical issues and the cost associated with their care. The RhCMV model is the primary non-human primate model for studying HCMV pathogenesis and is providing much insight into the areas of CMV disease in the context of HIV/SIV infection and vaccine development. Much like HCMV, RhCMV is extremely widespread with estimates of nearly 100% of Rhesus macaques (RM) kept in primate centers testing CMV positive (271). While this is an interesting correlate to HCMV, such widespread dissemination of RhCMV within the colony makes studies of primary infection very difficult. Again, much like what is observed in humans, infection of RM

typically results in an asymptomatic lifelong association. While major advances to the understanding of the pathogenic outcomes associated with HCMV including fetal infection and vasculopathies, along with the underlying mechanisms of the viral life cycle *in vivo* have been achieved with the use of small animal models there remains an imperative to validate the findings of small animal models within primates, to better predict outcomes in humans.

4. CELLULAR TROPISM

Numerous studies have detailed the wide variety of cell types that CMV can productively infect including: macrophages (Macs), endothelial cells (ECs), fibroblasts, stromal cells, epithelial cells, neuronal cells, hepatocytes and smooth muscle cells (SMCs) (233, 242, 249). HCMV infection of endothelial cells, macrophages/monocytes, epithelial cells and smooth muscle cells has been tightly associated with viral pathogenesis, immune evasion, dissemination and latency/persistence (reviewed in (241). Infection of both ECs and SMCs has been linked to the acceleration of vasculopathies, such as atherosclerosis and transplant vascular sclerosis. Fibroblasts are highly permissive for HCMV and likely contribute greatly to the total viral load. Infection of endothelial cells is believed to play a role in viral dissemination and persistence. Infection of cells of the myeloid lineage has been tied to the establishment of latency and the alteration of the adaptive immune response (19, 68, 112, 113, 115, 130, 170, 171, 202, 248, 281). Special attention has been paid to CMV infection of

endothelial cells and macrophages in the literature and as such is the focus of the next section.

Viral Mechanisms of Macrophage and Endothelial Cell Tropism

Numerous cellular and viral factors mediate CMV cell tropism. Viral determinants of cellular tropism may function in a variety of tasks including: entry, replication, virion morphogenesis, inhibition of apoptosis and viral egress. The ability of HCMV to infect and replicate in selected cell types, particularly macrophages and endothelial cells, has been found to be highly dependent on the strain/passage history of the virus (55). Lab adapted strains are attenuated or completely deficient in growth in these cell types compared to clinical strains. Further evaluation of the genomic composition of lab adapted strains revealed largescale deletions and point mutations ORFs mediating tropism.

a. Macrophage tropism

Because of the pivotal role that macrophages are believed to play in the biology of CMV infection including their likely role in dissemination and latency, much attention has been focused on understanding the viral determinants of macrophage tropism. One theme that emerges throughout the macrophage tropism literature is the finding that Macs are not a homogenous population of cells; their variable susceptibility to CMV infection appears to be directly linked to

both the maturation state and the differentiation pathway taken (78, 248). While seven MCMV and HCMV ORF gene products have been shown to mediate macrophage tropism, only one ORF product has been shown to facilitate viral replication in both mature and immature, primary and immortalized macrophages (96).

One of the first, and to date the most comprehensive study examining the role of macrophage tropism *in vivo* identified three ORFs mediating macrophage tropism: m139, m140 and m141 (96). Large scale deletion mutants lacking the HindIII J and I regions of the MCMV genome exhibited a severe growth defect in the differentiated macrophage cell line, IC21 (35). Fine mapping of the region isolated m139, m140 and m141 as the ORFs responsible (96). Further investigation *in vivo* revealed a significant reduction in viral titers in the macrophage dense spleen. Interestingly, the specific depletion of splenic Macs both restored the deletion mutant titers to wild type (intact splenic macrophage) levels and enhanced the overall titer of wild type MCMV as compared to replication in intact spleens, suggesting an overall net protective role for macrophages (90, 96). Follow up studies have demonstrated that m139-141 are not required for replication in more mature macrophages such as, primary exudate cells or J774A.1 (35, 162).

M45 was identified as a macrophage determinant in a studies using a macrophage cell line, IC21 (30). Although a growth defect in ECs infected with

an M45 mutant virus has been linked to an anti-apoptotic function, the mechanism of M45 mediated macrophage tropism has yet to be determined empirically.

M36, the next macrophage tropism determinant to be uncovered, is the only ORF reported that mediates replication in both primary and cell line macrophages of differing maturity (162). M36 (a homologue of the HCMV US22 family of genes) was identified by Menard and colleagues in a screen for replication in macrophages. M43 was also recognized as a macrophage growth determinant in the same screen. However, M43 only exhibited a significant growth defect in IC21, while M36 was defective for replication in all macrophage cell lines and primary cells tested. While the means by which M43 mediates growth in IC21 is still an unknown, M36 was found to inhibit apoptosis in infected macrophages (162). This anti-apoptotic function was not unexpected since Skaletskaya and colleagues had previously characterized UL36 as an inhibitor of Caspase 8 (243).

b. Endothelial cell tropism

Much like macrophages, the role of endothelial cells in CMV pathogenesis is not fully understood. However, several observations, including the finding that ECs are one of the predominately infected cell types during acute infection, point to a primary role for ECs in the life cycle of the virus (228, 242). In particular multiple studies implicate ECs in CMV pathogenesis, persistence and dissemination.

The virus has been detected in the endothelial lining of arterial vessel walls in HCMV seropositive patients not undergoing an active infection (102). Early studies by Myerson and colleagues found that ECs often can be infected without cytopathic effect (180). This phenomena was more closely examined in a study published in 1998 where HCMV infection of primary *macro*vascular aortic endothelial cells (AECs) was compared to that of brain *micro*vascular endothelial cells (BMVECs) (69). Fish *et al.* found that while both types of ECs supported a productive HCMV infection, infection of BMVECs resulted in a rapid lytic course with a more than 1000 fold higher titer of intracellular virus as compared to AECs which produced virus at much lower levels and without significant cytopathic effect, thus implicating ECs as a possible reservoir for persistent HCMV (69). The observation of clumps of HCMV positive endothelial cells in the bloodstream of patients actively infected lends credence to the hypothesis that ECs may also function in viral dissemination (81).

To date five ORFs have been identified in MCMV and HCMV as endothelial cell tropism determinants: M45, UL24, UL128, UL130, UL131 (30, 87). The first to be identified, M45, is the only MCMV EC tropism determinant described in the literature (30). Random transposon mutagenesis of the MCMV genome revealed a growth defect in ECs but not fibroblasts infected with an M45 insertional mutant (30). M45 encodes a ribonucleotide reductase sequence homologue, however the MCMV gene product does not appear to be a functional homologue, but rather serves an anti-apoptotic function (30, 140). In contrast the corresponding

HCMV sequence homologue, UL45, is dispensable for growth in human umbilical cord vascular endothelial cells (86). It is still not clear if the difference in tropism seen with MCMV and HCMV homologues is a species specific divergence in function or a cell type specificity, as previously observed in the different replication patterns of HCMV in micro- versus macrovascular endothelial cells (69).

More recently Hahn and colleagues mapped HCMV endothelial tropism to the UL128, 130 and 131 ORFs (87). Repair of a single nucleotide insertion present in the AD169 strain UL131 ORF, was able to rescue AD169 infection of both epithelial and endothelial cells (295). Deletion of this region of the viral genome results in a blockade in receptor mediated endocytosis of the virus. Ryckman and colleagues found that UL128, 130 and 131 each independently and in concert form a complex with the viral glycoproteins gH/gL, viral glycoproteins known to mediate viral entry (227). Expression of UL128, 130 and 131 together resulted in the dramatic increase in gH/gL export to the cell surface, an indicator of incorporation into the virion, and a requirement for infection.

Relatively less is known about the remaining ORF implicated in EC tropism, UL24. Dunn and colleagues identified UL24 as an EC tropism determinant in a global screen of the HCMV genome in which individual ORFs were knocked out and the resulting mutant viruses were tested for growth on various cell types (55). HCMV ΔUL24 displayed a nearly three log reduction in peak titers compared to

the wild type parental virus in human microvascular ECs. The precise means by which UL24 mediates EC tropism is still an open question. The ORF encodes a tegument protein and as such is assumed to play a role in virion morphogenesis in ECs, but as yet this has not been demonstrated empirically (4).

5. LATENCY AND PERSISTENCE

While productive infection with Human cytomegalovirus is typically resolved by the host immune system, the virus is not cleared and remains in a latent or persistent state throughout the life of the host. The extensive cell and tissue tropism of CMV has obscured our understanding of the mechanisms of CMV latency and persistence. With an abundance of cellular targets, each with apparently different infection patterns *in vivo* (as determined by transcriptional patterns and dependence on differentiation status), questions involving latency and persistence are vastly complex and require a clear definition of both latency and persistence as they pertain to viral infections in general and specifically CMV.

a. Latency Versus Persistence

The classical definition of viral latency is a state of infection characterized by the presence of the viral genome, absence of infectious virus and the ability to reactivate viral replication (70). The ability to reactivate differentiates a latent state from an abortive infection of a non-permissive cell type. The term

persistence, however, is commonly used to describe a state of low-level productive viral replication that is not cleared by the host immune system. One of the perpetual debates in the study of CMV latency versus persistence is the question of whether latency and persistence pertain to the organism as a whole versus at the level of the single cell. While it is clear that CMV establishes a latent infection at the cellular level, deciphering whether or not the virus establishes a truly latent infection of the organism as a whole is hampered by technical challenges relating back to detection limits of infectious virus.

Reducing the question of latency or persistence to the cellular (versus organismal) level provides a framework within which to ask the pertinent questions regarding the mechanisms of CMV establishment of latency and reactivation, including the basic questions pertaining to the reservoirs for latent virus and the conditions required for the establishment and maintenance of latency and reactivation. Within this framework, there is evidence to suggest that while the virus is persistent in some cell types, the virus may remain latent in others.

b. Proposed Reservoirs

While a variety of cell types have been proposed to be reservoirs for persistent or latent CMV, one cell type stands out in the literature as the most likely candidate for latent CMV reservoir: a myeloid progenitor. The first evidence to suggest that

cells of the myeloid lineage may act as sites of latency was the finding that the leukocyte fraction of the peripheral blood can transmit HCMV from healthy virus carriers to previously seronegative recipients (10, 136, 263). Viral DNA, but not infectious virus, is readily detectable in this fraction of the blood.

Several studies have suggested multiple reservoirs of persistent virus infection, including endothelial cells, bone marrow stromal cells (BMSC) and the ductal epithelial cells of the submandibular gland (69, 129, 240, 283). Aortic endothelial cells (AECs) have been suggested as a possible persistent reservoir due to the long term productive, non-lytic infection observed in HCMV infected AECs (69). Bone marrow stromal cells have been shown to be permissive to HCMV infection and are thought to be a source of infectious virus for myeloid progenitors (240, 283). Utilizing the RCMV model in combination with immunohistochemistry, *in situ* hybridization and electron microscopy of SMG taken from infected rats Kloover *et al.* identified striated ductal epithelial cells as exclusive sites of RCMV persistent virus localized to the SMG, a finding supported by the observation that after primary infection animals and humans continue to shed virus in the saliva (129).

c. HCMV Reactivation Models

The species specificity of the CMVs necessitates the development of *in vitro* models to study the requirements of HCMV latency and reactivation. Several

models have proven highly effective at answering some of the basic questions concerning latency (reviewed in (266).

The Söderberg-Nauclér model, designed to address questions related to HCMV reactivation from latency, examined mechanisms of reactivation in CD14+ monocytes infected *in vivo* (247). The previous monocyte derived macrophage (MDM) differentiation method developed by Ibanez *et al.* to support the productive replication of HCMV in monocyte derived macrophages infected *in vitro* was unable to support reactivation from latency in PBMCs taken from infected individuals (275). Studies investigating MCMV had found that allogeneic stimulation of peripheral blood mononuclear cells did induce productive viral replication in previously quiescent cultures (187, 232). Söderberg-Nauclér *et al.* expanded on the observation that reactivation of MCMV can be triggered by immunosuppression and donor/recipient histoincompatability and hypothesized that reactivation required a unique MDM differentiation pathway to reactivate from latency (32, 91, 211, 232, 310). Peripheral blood derived monocytes isolated from seropositive asymptomatic healthy donors were able to reactivate from latency when stimulated to differentiate *in vitro* by allogeneic stimulation, providing a better model for transplant induced reactivation (247).

The most recently developed *in vitro* latency model addressed the question of progenitor phenotype and the relationship with the establishment of latency (78). While CD34+ progenitor cells had previously been identified as a CMV+

reservoir, these cells represent a heterogeneous population including pluripotent stem cells and cells in the early phases of lineage commitment, marked by the onset of CD38 expression and concomitant decrease in c-kit expression (78). In these studies Goodrum and colleagues utilized CD34+/CD38- and CD34+/CD38+ cells isolated from bone marrow, infected them *in vitro* and then co-cultured the infected quiescent cells with immortalized fibroblasts. Infectious center assays revealed that the more stem cell like CD34+/CD38-/ckit+ cell was more able to reactivate infectious virus, whereas committed stem cells were not able to reactivate. This finding suggests a lineage commitment already divergent from that used by the virus to reactivate.

B. IMMUNE RESPONSE TO CMV INFECTION

While CMV infection of the immunocompromised host culminates in severe pathology, infection of the immunocompetent host is typically asymptomatic. The difference in disease outcome can be attributed to the substantial response mounted by the immune system of the competent host. Resolution of primary CMV infection in the immunocompetent host is due to the coordinated action of both the innate and adaptive arms of the immune response. Innate immunity, primarily interferon (IFN) and natural killer (NK) cell mediated responses, is critical to the early control of viral infection, while the adaptive immune response controls the later stages of infection. Those adaptive immune responses providing the greatest degree of protection appear to be T-cell mediated. Both T-

cell and antibody responses are likely to contribute to the control of recurrent infections derived from persistent infection, reinfection or reactivation from latency. While most of what is understood about the immune response to CMV is derived from MCMV model studies (131, 150, 210), clinical reports of HCMV infection appear to parallel much of what has been observed in animal models of *in vivo* infection.

1. INNATE IMMUNE RESPONSE

a. Interferon Response

Interferons are a subset of cytokines with antiviral effector properties and represent one of the major contributing arms of the innate response to viral infection. First recognized in 1957 for their ability to induce an antiviral state, the complex web of downstream effector proteins induced by IFNs provide a formidable challenge to viral replication (109, 181). Three main functions can be attributed to the IFN response: host protein synthesis shut down, an increase in antigen presentation and the activation of Natural Killer Cell (NK) cytolytic activity.

Interferons are subdivided into two groups Type I and Type II (317). Type I IFNs include IFNα and IFNβ. Their synthesis is induced by signaling through Toll-Like Receptors, invariant pattern recognition receptors which bind a variety of protein and lipid motifs common to invading pathogens (17). IFNα comprises a family of

proteins, primarily expressed in leukocytes; while IFNβ is encoded by a single gene and is expressed by most cell types (317). Synthesis of Type I IFN in infected cells can be induced by dsRNA. These secreted effector proteins act in both an autocrine and paracrine manner through their interaction with the IFN receptor.

Interferon γ (IFNγ) is classified as a Type II IFN (317). Production of IFNγ in NK and T cells is stimulated by the engagement of the T Cell Receptor (TCR) or NK cell Receptor (NKCR). Engagement of the IFNγ receptor stimulates a signal transduction cascade culminating in the induced transcription of genes involved in antigen presentation and antiviral proteins iNOS (inducible nitric oxide synthase) and IDO (IFNγ induced indoleamine 2, 3-dioxygenase) (317). In addition to their individual roles in the antiviral response, both Type I and Type II IFN have the net effect of stimulating both the adaptive and innate immune response through the upregulation of antigen presentation and the activation of NK cell effector functions (317).

Role of IFN in CMV Infection

The importance of IFN-mediated control of CMV infection *in vivo* has been tested in a multitude of ways using the MCMV model. Several studies have demonstrated a diminished CD4 and CD8T cell response in mice that have been IFNγ depleted and then infected with MCMV (104, 153, 201). Lucin *et al.* found

that IFNγ is required for normal viral clearance from the SMG (153). Hengel *et al.* observed a dependence on IFNγ for efficient MHC I presentation of viral peptides to CD8T cells (104). Later studies also documented a dependence on IFNγ for efficient antigen processing *in vivo* (76). Furthermore, a study utilizing IFNγ receptor knock out mice found that IFNγ played a role in the clearing acute infection (204). Those mice which were unable to signal through the IFNγ Rc maintained a persistent chronic infection long after congenic control mice had cleared the virus (204). Mice treated with IFNγ prior to infection with MCMV were protected from lethal infection (65). The prophylactic effect of IFN pretreatment was mirrored in a clinical study in which patients scheduled to receive renal transplants were treated with IFNα before surgery (106). Those pretreated with IFNα had reduced clinical signs of HCMV disease (106). A common theme to all of the afore mentioned studies is the limited effectiveness of IFN treatment post-infection, even treatment administered at the time of infection has little impact on the outcome of viral infection. While IFN impacts the course of CMV infection *in vivo*, the effect is early and can be countered by the virus (103, 104, 152).

b. Natural Killer Cells

Natural Killer cells (NK) are large granular cells originating from a lymphoid progenitor shared by B and T cells. Although these cells are of lymphoid origin, NKs lack the antigen specific receptors expressed in lymphocytes and as such are generally grouped within the innate arm of the immune response (111). Two

broad classes of invariant receptors, inhibitory and activating, are expressed on the cell surface, and refer to their respective abilities to either inhibit or activate the cytolytic response of NKs (208). The balance between signals received from these two classes of receptors regulate NK cell effector functions (208). Although antigen specific receptors are absent on the surface, NKs are able to recognize cells that have transformed and those that have been infected with intracellular pathogens, herpesviruses in particular, through an assortment of invariant receptors that are able to distinguish between self and "altered self" (194). Recognition of transformed or infected cells signals the NK cells to release cytotoxic granules on the surface of the target cell (208). These effector proteins penetrate the cell and induce apoptosis.

Role of Natural Killer cells in the immune response to CMV infection

Multiple studies support the critical role of Natural Killer cells in mediating early control of CMV infection in both the murine model and in the context of human infections. One of the earliest clues to the importance of NK control of MCMV infection was made apparent during a study investigating the effects of the beige gene mutation, conferring a selective NK deficiency, on the course of MCMV infection *in vivo* (236). Shellam and colleagues observed that mice homozygousfor the beige gene were much more susceptible to lethal infection than their NK sufficient littermates (236).

In addition to the mere presence of NK cells, Bancroft *et al.* found that mouse strains with highly active NK cell populations are more resistant to MCMV infection (13). Further evidence supporting the significance of NK mediated control of CMV infection *in vivo*, can be found in NK depletion experiments in which the selective loss of NK cells resulted in greater dissemination of virus, six to eight fold higher SMG titers, increased severity of pathogenesis including splenic necrosis and lung leucopenia (33). Bukowski *et al.* found that the differences in severity of infection was greatly dependent on the timing of NK depletion, and that early loss of NK cells mediated the greatest reduction in immune control, suggesting a role for NKs prior to the adaptive immune response (33). The critical nature of NK mediated control was further underscored in 2001 when three different groups traced the genetic resistance factor (known previously as *cmv1*^{r}) responsible for C57Bl6 strain resistance to MCMV to the gene encoding the NK activating receptor, Ly49H (27, 48, 139). Those strains of mice with intact Ly49H receptors were shown to resist MCMV infection. NK cells are vital to immune control of acute MCMV infection limiting both viral dissemination and the degree of pathology associated with infection.

While the significance of NK mediated control of HCMV is less apparent than in the MCMV model system several lines of evidence suggest the critical role of NK cells in immune control of HCMV. Remarkably, a case report of a patient diagnosed with a specific deficiency in NK cells presented with recurrent uncontrolled HCMV infection (16). Although not an indisputable link, the

syndrome does intimate a key role for NK cells in HCMV infection *in vivo*. More suggestive evidence that NK cells are critical in controlling HCMV infection is illustrated by the considerable number of HCMV encoded gene products that have been demonstrated to block NK cell recognition of infected cells (170). Such a large proportion of the genome dedicated to manipulating NK function and recognition of infected cells points to selective pressure on the virus to evade the NK response. These ORFs and their gene products, as well as those encoded by MCMV, will be discussed in greater detail in the immune evasion section.

2. ADAPTIVE IMMUNE RESPONSE TO CMV INFECTION

The adaptive immune response initiates when a foreign antigenic peptide presented in the context of MHC and costimulatory molecules is recognized by the antigen-specific T cell and subsequent activation of the antigen-specific B cell (193). Primary infection of the immunocompetent host induces profound CD8 and CD4T cell responses, despite which the virus is still able to maintain a lifelong infection of the host. While CMV infection of the immunocompetent host is characteristically asymptomatic due to the efficient control of viral replication and subsequent viral-induced pathogenesis, the interplay between the host immune response and CMV is a lifelong battle. The investment that the host immune system makes toward control of CMV replication has only recently come to light. Utilizing nearly 14,000 overlapping peptides found in over 200 HCMV ORFs Sylwester *et al.*, found that 151 HCMV ORFs elicit CD4 and or CD8T cell

responses and that these total nearly 10% of the entire host memory T cell compartment (272). While the adaptive immune response in the healthy host clearly minimizes overt pathology, the staggering T cell response does not however induce sterilizing immunity (5, 22).

a. CD8T cell response

Effector T cells can be broadly divided into three functional subclasses based on their capacity to recognize peptide:MHC I or peptide:MHC II complexes and the subsequent cytokine response elicited by the interaction. CD8T cells, commonly referred to as cytotoxic T cells (CTL), express the CD8 coreceptor which in combination with the T cell receptor (TCR) recognizes peptide antigens displayed in the context of MHC I molecules [FIGURE 3]. In general, peptides which are displayed on MHC I molecules are derived from cytosolic proteins including peptides generated from proteins synthesized by intracellular pathogens. The primary effector function attributed to cytotoxic T cells is the recognition and elimination of infected host cells, either through direct lysis or induced apoptosis (111, 193).

The importance of the adaptive cellular immune response in controlling HCMV infection can be inferred from the severity of CMV induced disease in those individuals with impaired T and B cell responses following transplantation. The role of T cells in protection from CMV disease has been more clearly defined in

the mouse model, where adoptive transfer of MCMV-specific CD8T cells was both shown to protect against a lethal dose of MCMV and a combined deficiency in CD8 and CD4T cells was demonstrated to have a dramatic effect on the reactivation of latent infection (200, 201, 212). These findings support a dual role for T cell control of CMV in both limiting disease course due to primary infection and minimizing reactivation from latency.

b. CD4T helper cell response

CD4T cells are defined by the presence of the CD4 coreceptor, integral in the recognition of antigenic peptide:MHC II complexes. While recent studies have demonstrated the existence of both cytotoxic and helper CD4T cells, the contribution of cytotoxic CD4T cells to CMV immunity is a burgeoning field and more is understood about the contribution of helper T cells (T_H) in the context of CMV immune responses (287). The moniker *helper* refers to the intrinsic ability of CD4T cells to stimulate B cell, CD8T cell, monocyte and macrophage effector functions (111, 194). Proliferating CD4T cells differentiate into one of four types of T_H effector, T_H1, T_H2, iTreg, and T_H17, depending on the cytokine microenvironment and the quality of the MHC:peptide:TCR interaction (111, 313). Those helper responses which stimulate cell-mediated immunity are collectively referred to as T_H1.

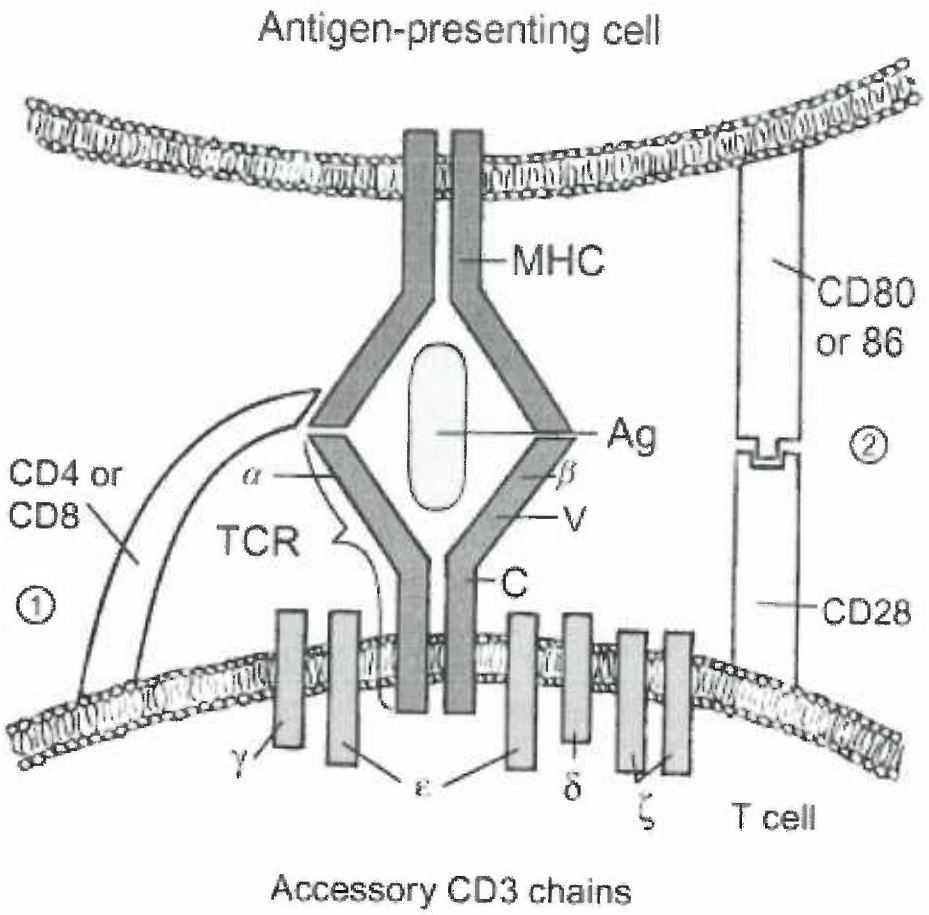

Figure 3. ANTIGEN PRESENTATION TO T CELLS

Two signal model of T cell activation

The TCR, comprised of one a and one b chain bind to antigen:MHC complex on the antigen presenting cell. CD4 or CD8 molecules present on the T cell interact with MHC II or MHC I molecules (respectively). This provides the first signal. Interaction between CD28 on the T cell and CD80 or CD86 on the APC provides the second costimulatory signal.

Adapted from Merck Manuals Online Medical Library

The activation of macrophages and induction of CD8T cell proliferation by T_H1 cells is due in large part to the secretion of IFNγ and induced expression of IL12 in DCs (45, 154). The type of T_H responses inducing humoral immunity are termed T_H2 (111). The production of IL4, IL13, IL5, IL6 and IL10 by T_H2 cells stimulates B cell proliferation, induces antibody class switching and increases antibody production (45, 154). Both T_H1 and T_H2 cytokine responses act to promote their own function in part by inhibiting the actions of the other (*i.e.* T_H1 responses inhibit T_H2 responses and vice versa). Thus commitment to the T_H1 or T_H2 lineage appears to be a major fork in determining the quality of antiviral response elicited by the virus. Recent studies investigating immune control of chronic viral infection have highlighted the pivotal role of the T_H1 versus T_H2 response in promoting either viral clearance (T_H1) or chronic infection (T_H2) as seen in Lymphocytic Choriomeningitis Virus and Hepatitis C (56, 67).

Researchers are just now beginning to understand the degree and significance of the CD4T cell response to CMV control. Technical challenges to studying the response have been limited by the available methods. A conservative estimate of the degree of CD4T cell involvement in CMV immunity was recently defined utilizing intracellular cytokine staining of CD4T cells treated with a panel of CMV-derived peptides spanning more than 90% of the viral ORFs (272). IFNγ production in response to peptide stimulation was used to assist in defining a CMV-specific memory phenotype in more than 9% of the total CD4T memory

population (272). The size of the memory CD4T cell population mirrored that of the CD8T cell population (272).

While the precise level of protection provided by CD4T is still being elucidated, several key studies coupled with our basic understanding of T cell biology suggest a key role. Gamadia *et al.*, uncovered a correlation between the onset of disease symptoms and the timing of the CMV-specific CD4T cell expansion (75). This group found that in primary infection of renal transplant patients symptomatic HCMV infection was closely associated with a delayed CD4T cell response, despite equivalent CD8T cell populations and phenotype (activated effector). The primary finding of the Berge group was that despite the presence of both a functional CD8T and antibody response, those individuals with a delayed or absent CD4T cell response had a much higher incidence of symptomatic HCMV compared to those with early CD4T expansion. Studies involving the mouse model have further underscored the importance of the T helper contribution to protective immunity. Polic *et al.* used a B cell deficient mouse model to define a hierarchy of protective T cell and NK responses in MCMV latency/reactivation (201). In the absence of B cells, it was found that CD8T cells provided the greatest control of viral load upon reactivation, followed by NKs and CD4T. Given the role of CD4T in promoting B cell responses, it is likely that the contribution of CD4T toward control of total viral load in the reactivation model may have been underestimated.

c. B cell response

B cells are derived from hematopoietic stem cells which differentiate in the fetal liver and adult bone marrow. The B cells recognize antigen through the B cell receptor (BCR), a cell surface immunoglobulin. Upon activation, the B cell is stimulated to further differentiate and produce antibody molecules of the same specificity as the BCR. With regard to viral infection, antibodies function to block viral entry into the cell, through the interaction of neutralizing antibodies with the viral glycoproteins. Antibodies can also trigger the activation of Macs and NK cells by coating infected cells displaying viral antigen at the cell surface with non-neutralizing antibody, a process known as opsonization (111). Antibody responses can provide long term immunity against various viral pathogens, however the antibody response elicited by CMV is inefficient at inducing sterilizing immunity.

Although CMV induces both neutralizing and non-neutralizing antibodies the response is unable to block recurrent infections (211). Additionally, studies investigating the B cell contribution to resolution of primary infection in mice, found no difference in B cell-deficient mice compared to control mice (119). However, transfer of polyclonal antibody has been demonstrated to protect against a lethal dose of MCMV in naïve recipients (207, 235). Likewise, adoptive transfer of memory B cells in T and B cell deficient mice was also shown to dramatically reduce viral loads and protect against a lethal challenge (128). While

antibodies fail to block recurrent infections they do play a protective role in several settings, including the prevention of primary infection and control of viral reactivation.

3. DENDRITIC CELLS ARE THE BRIDGE BETWEEN THE INNATE & ADAPTIVE RESPONSES

a. Dendritic Cell Origin, Phenotype and Function

Dendritic cells (DCs) comprise a heterogeneous population of antigen presenting cells which in their immature state act as sentries in the periphery. Upon encountering a pathogen, DCs mature and migrate to secondary lymphoid organs where they present antigen derived from the offending pathogen in the context of major histocompatability molecules I and II (MHC I and MHC II) in combination with costimulatory molecules, such as CD80 and CD86.

DCs are derived from several different bone marrow progenitors, each with unique functional characteristics highly dependent upon the maturation status of the DC. Although our understanding of DC origin and function is still evolving, currently three different pathways detailing DC differentiation from distinct bone marrow precursors under the influence of various cytokine cocktails and tissue environments have been described: myeloid, lymphoid and plasmacytoid DCs.

Myeloid DCs, include the epidermal resident Langerhans Cells (LCs) as well as circulating DC and DCs in the peripheral tissues. This class of DC is of myeloid origin and can be differentiated *in vitro* from peripheral blood monocytes cultured in GM-CSF and IL4. Myeloid DCs are generally CD14 negative, CD1a positive, HLA-DR and HLA-DQ high (122, 273). Several studies have demonstrated that myeloid DCs tend to preferentially prime a T_H1 response (105, 159).

DCs classified as lymphoid tend to be CD11b and CD14 positive and CD33 negative. These cells are typically found in the lymph nodes and thymus and tend to elicit a T_H2 versus T_H1 response in the cognate T cell. Stimulation of a thymic progenitor *in vitro* with IL-3 or CD40L in combination with IL2 and IL15, but not GM-CSF leads to the generation of lymphoid DCs. This finding suggests that lymphoid DCs share a precursor in common with T cells.

Plasmacytoid DCs (pDCs) possess a round shape morphologically similar to plasma cells and can be found in the blood, spleen, thymus and bone marrow (reviewed in (308) (60, 239) (8, 39, 270). PDCs express MHC II, CD45RA and T cell markers (including CD4); they tend to be CD11c and CD11b negative. Due to the expression of T cell markers and the ability to stimulate pDC differentiation *in vitro* from thymic precursors, pDCs were originally believed to be of lymphoid origin, but a recent study suggests both a common lymphoid progenitor and a common myeloid progenitor (311). PDCs secrete copious amounts of IFNα and IFNγ (308).

b. Enhancement of the Innate Immune Response

Although most commonly associated with activation of adaptive immunity, dendritic cells are also potent stimulators of the innate immune response, particularly natural killer cells. Direct contact between DCs and NKs leads to the secretion of IL12, IL18 and IL2 by DCs (80). The cytokine cocktail released by the activated DC stimulates NK proliferation, IFNγ production and enhances the cytotoxic capacity of the NK cell (66).

c. Dendritic Cells Trigger the Adaptive Immune Response

While DCs of different origin possess specialized traits (varied cell surface marker expression and function), the common characteristic of all DCs is the innate ability to effectively prime the T cell response. DCs circulate in the blood as immature precursors; migration into peripheral tissues stimulates differentiation. These tissue resident immature DCs actively take up and process antigen, presenting it on the cell surface in the context of major histocompatability complexes (MHC I and MHC II). Upon receipt of the appropriate signals, DCs in the periphery undergo maturation and migrate to secondary lymphoid tissues. It is in the lymphoid organs that DCs present antigen in the context of MHC I and MHC II to circulating T cells. Activation of naïve T cells specific for the presented antigen requires both a primary (MHC:TCR) and secondary (costimulatory) stimulus. Upon receipt of both signals naïve T cells proliferate and differentiate

into effector T cells. Alternatively if the DC fails to be activated (absence of costimulatory signal) the DC induces T cell tolerance to the antigen presented.

Although historically DCs were believed to stimulate the antibody response solely through CD4T helper cell activation, recent evidence points to both an indirect and direct involvement of DCs in B cell activation (53, 111). In addition to the indirect role of DC stimulation of CD4T helper activation, DCs have been demonstrated to directly enhance proliferation, antibody secretion and isotype switching in naïve B cells (53, 63).

Specific evidence of DC involvement in the protective immune response to CMV infection can be found in the MCMV model. Several studies by Dalod *et al.*, demonstrated that upon an *in vivo* challenge with MCMV DCs are induced to mature, increase cytokine production and activate NK cells (46, 47). Additionally, DCs isolated early post *in vivo* infection were found to efficiently activate CD8T cells. Further investigation of subset specialization found that plasmacytoid DCs isolated from infected mice produced elevated levels of IFNα/β, MIP1α, TNFα and IL12, while expressing reduced levels of MHC II and costimulatory molecules relative to other DC subsets (46, 47). This finding points to a probable role in activation of the innate immune response by pDCs. Furthermore the importance of DCs in the antiviral response to CMV infection is highlighted by the acute susceptibility of TLR9 deficient and TLR3 KO mice to MCMV infection. Toll-like receptors 9 and 3 are expressed by DCs and are responsible for detection of

pathogens. TLR3 KO mice and those deficient for TLR9 have elevated viral loads and a diminished capacity for NK and NKT cells to respond to infection (274).

d. Dendritic Cells as Targets for Infection

While a variety of cell types are able to present antigen, both constitutively and upon induction, dendritic cells are widely recognized as the most potent antigen presenting cells. Their ability to express both MHC I and MHC II in combination with costimulatory molecules (CD80, 86) together with their capacity to home to lymphoid organs (upon encountering a pathogen) where they present antigen to circulating naïve T cells, all contribute to their unique ability to effectively prime the T cell response (111). Dendritic cells are responsible for the induction of the adaptive and enhancement of the innate immune response; this points to their pivotal role in the induction of an effective antiviral response. The ability to infect and subsequently manipulate the function of DCs is a huge advantage to Cytomegaloviruses and mechanisms of CMV-induced paralysis of DC function will be discussed in greater detail in the immune evasion section.

Viral DNA detected in DCs isolated from viremic kidney transplant patients (14) and RNA and antigen detected in circulating blood DC taken from heart transplant patients has provided evidence of HCMV infection of DC *in vivo* (289).

In further support of dendritic cells as a natural target of CMV infection *in vivo*, DCs isolated from mice infected with an MCMV mutant virus expressing the LacZ gene were found to express the enzyme (9).

Several clear advantages to viral infection of DCs are readily apparent. First, DCs are long lived cells and as such could serve as a prime reservoir for latent or persistent virus. Second, DCs traffic throughout the body and so could potentially provide an apt vehicle for dissemination. Third, the inherent ability of DCs to prime both the adaptive and innate immune response make the infection and subsequent manipulation of DC function a key advantage to the virus. While possible links to latency or dissemination have yet to be proven experimentally, the effect of CMV infection on the ability of DCs to function normally has been demonstrated in several studies (38, 176, 177, 205, 206, 289, 290).

C. CMV IMMUNE EVASION

While the HCMV genome encodes more than 180 predicted ORFs, only one third of those have been identified as essential for viral replication *in vitro* (55, 312). It is estimated that nearly two thirds of CMV encoded proteins are dedicated to sustaining viral infection *in vivo*. These *in vitro* accessory gene products are likely essential to viral maintenance *in vivo*: facilitating viral dissemination, reactivation from latency, host cell survival, immune evasion and manipulation.

1. EVASION OF THE INNATE IMMUNE RESPONSE

a. Interference with the IFN Response

Interferons are potent antiviral cytokines, which both initiate innate resistance and stimulate the adaptive immune response to viral infection. Studies utilizing a comparison of inactivated versus replication competent virus have clearly demonstrated that CMV takes an active role in disarming the IFN response (29). Viruses within the Cytomegalovirus genus have evolved a number of ways to counter the IFN response at three different points in the pathway: IFN induced gene transcription, IFN receptor signaling and IFN induced effector proteins (29).

Interference with Type I IFN induced gene transcription

Evidence of a blockade in various IFN-inducible genes was observed using HCMV-microarray analysis of UV-inactivated versus infectious HCMV. The transcript analysis found that infection with replication competent virus resulted in the down regulation of IFNβ, IRF1, IL6, human inducer of IFNγ (HuMIG), MIP1α, MIP3β, RANTES (29). Later, a structural component of the virion, pp65 (UL83) was found to mediate the down regulation of a specific subset of IFN-induced genes, including MxA, ISG-16K and IFNβ (2, 28). In addition to pp65 mediated depletion of IFNβ transcription, the full affect of HCMV induced down regulation of IFNβ transcription was found to be dependent on *de novo* viral gene expression.

Studies executed in the Bresnahan laboratory identified the viral gene product IE86 as a potent IFNβ antagonist (276).

Interference with IFN Receptor Signaling

Another means by which CMV inhibits the IFN response is by blocking IFN receptor signal transduction. Once IFNs bind their cognate receptor, a cascade of downstream signaling events begins. Although Type I and Type II IFN have distinct signaling pathways, both types utilize Jak/STAT (Janus kinase signal transducer and activator of transcription) signaling as a key point in their separate signal transduction pathways (1, 125).

A blockade in STAT1 tyrosine phosphorylation and subsequent nuclear translocation has been observed late (72hpi *in vitro*) in HCMV infection (137). Although an ORF responsible for this effect has yet to be identified, it is believed that a drop in Jak1 protein levels observed by Miller *et al.* is likely responsible for the inhibition of STAT1 activation (167). Again, to date no ORF has been linked to the drop in Jak1 protein levels. Recently Paulus *et al.* discovered a link between HCMV IE72 protein and STAT2 that resulted in a blockade of ISG induction (195).

MCMV has also been demonstrated to interfere with Jak/Stat signal transduction, primarily through the action of a protein encoded by the M27 ORF (316).

Zimmermann and colleagues identified pM27 in a phenotypic screen of MCMV mutants generated by random transposon insertion. An ISRE reporter construct was used to identify ORFs involved in IFN blockade. Although M27 is not essential for viral replication *in vitro*, Abenes *et al.* have shown a dependence for replication *in vivo* (3). The essential role of pM27 for *in vivo* replication can be attributed to the virus's unique ability to target both the Type I and Type II IFN response by directly binding and down regulating STAT2 (316).

Interference with IFN induced effector proteins

Although CMVs are able to impede IFN induced gene transcription and IFN Rc signaling, viral blockade of these stages of the IFN response is not complete, and as such CMVs have evolved to also inhibit the downstream effector proteins of the IFN pathway. The effector proteins encoded by interferon-stimulated genes (ISGs) generally fall into two main categories: those that directly inhibit viral replication (most commonly through inhibition of transcription/translation) and those effectors involved in the immune response. One of the most widely targeted classes of ISGs includes those proteins involved in antigen presentation. CMV interference with antigen presentation is the focus of this thesis and as such will be discussed at length, in the antigen presentation section.

Protein kinase R (PKR) and 2', 5'-oligoadenylate synthetase (OAS) are examples of two ISGs whose downstream effector functions are inhibited by HCMV (88, 89,

221, 222). The ability of the virus to block PKR and OAS was first identified in an effort to define one or more ORFs mediating the effect of HCMV subversion of the IFN response. Child *et al.* executed an elegant study in which recombinant Vaccinia Virus (lacking the E3L ORF responsible for inhibiting dsRNA induced IFN response in VV) was complemented with an HCMV gene library and assessed for the recombinant virus's ability to rescue the E3L deletion (40). Using this method, the IRS and TRS1 ORFs were identified. Later studies demonstrated the ability of IRS and TRS1, both virion components, to bind dsRNA and TRS1 to sequester inactive PKR in the nucleus (88, 89, 221, 222).

b. Manipulation of NK Function/Recognition

The importance of NK cell mediated clearance of viral infection is underscored by the multipronged approach CMV takes to evade NK recognition. The virus has evolved to both suppress activating signals and stimulate inhibitory signals. More than 16 ORFs in MCMV and HCMV have been identified that manipulate NK cell recognition of CMV-infected cells.

Suppression of activating signals

Both HCMV and MCMV actively down regulate ligands for the activating NK receptor, NKG2D, thus evading NK mediated immune surveillance. The protein encoded by the HCMV ORF UL16 retains three of the six recognized ligands for

NKG2D (MICB, ULPB1 and ULPB2) in the ER (54, 220, 301, 307). More recently an HCMV encoded microRNA, miR-UL112, has been demonstrated to inhibit the translation of MICB (262). Likewise MCMV reduces the surface expression of three NKG2D ligands, MULT1, RAE-1 and H60, through the action of the proteins encoded by m145, m152 and m155 respectively (133, 134, 148, 149). Down regulation of NKG2D ligands was demonstrated to have a direct effect on the ability of NKs to recognize infected fibroblasts both *in vitro* and *in vivo*, thus confirming the significance of NKG2D-dependent recognition of CMV infected cells (133, 148). UL141 blocks surface expression of CD155, the ligand for NK activating receptors CD226 and CD96 (280).

In addition to NKG2D mediated activation of Natural Killer cells, antibody coated target cells are recognized through an NK surface Fc receptor. Engagement of the NK Fc receptor triggers the antibody dependent cell-mediated cytotoxicity (ADCC) response and results in the release of cytotoxic granules such as perforin and granzyme (111). Five ORFs in MCMV and HCMV are confirmed Fc receptor homologues, m138, UL119, UL118, TRL11 and IRL11 (11, 144, 277, 280).

Stimulation of inhibitory signals

Inhibitory receptors in mice include the Ly49 family of C-type lectins and the NKG2 family of receptors shared by both mice and humans. In addition to the NKG2 family, humans also express the KIR family, an immunoglobulin gene

superfamily member, of inhibitory receptors. Stimulation of inhibitory receptors is induced by the recognition of "self" in the form of MHC I molecules. The Ly49 and KIR family of receptors signal when bound to classical polymorphic MHC I molecules (HLA-A, B, C in humans). The NKG2 family proteins form heterodimers with CD94 which recognize nonclassical, nonpolymorphic MHC I (HLA-E in humans and Qa1 in mice) (111).

CMV stimulates NK inhibitory signals via three mechanisms: induced upregulation of host nonclassical MHC I, expression of viral MHC I homologues and a viral encoded ligand of inhibitory receptors. HCMV induces the surface expression of the nonclassical MHC I molecules, HLA-G and HLA-E (via gpUL40), ligands of inhibitory NK receptors (188, 279). Selective upregulation of nonclassical MHC I molecules enables CMV infected cells to evade "missing self" based NK targeting, while avoiding presentation of virally derived antigens that would be presented in the context of classical MHC I, thus evading CD8T cell detection.

An alternative approach to evading the "missing self" NK targeting is through the expression of decoy MHC I molecules. MCMV and HCMV both encode structural homologues of MHC I molecules. The protein encoded by UL18, the HCMV MHC I mimic, was shown *in vitro* to inhibit NK activation through its interaction with the NK inhibitory receptor LIR1 (216). Blockade of NK function *in vivo* was observed for the MCMV MHC I homologue, m144 (61). In addition to its capacity

to upregulate HLA-E, the HCMV protein encoded by UL40 also acts as a peptide ligand for the inhibitory CD94:NKG2A heterodimer (284).

c. Evasion of Complement Cascade

The complement system is comprised of a series of proteolytic, inflammatory and regulatory proteins which function in an enzymatic cascade culminating in the lysis or phagocytosis of the extracellular pathogen or infected host cell (111, 194). The three separate pathways comprising the complement system involve a series of cleavage reactions all converging on one step, the cleavage of C3 to C3a and C3b, generating the C3 convertase proteolytic enzyme. As part of the host cell regulation of complement activity, binding of C3b to the host cell can be competitively inhibited by the cellular proteins CD55 and CD46, both of which are upregulating in HCMV infected cells (259). Both CD55 and CD46 have been identified as virion components, suggesting an additional evasion strategy from complement mediated virion lysis (253, 258). In addition to the inhibition of C3 convertase activation, HCMV, MCMV and RCMV may interfere with complement binding to antigen-antibody complexes through the action of virally-encoded Fc receptors (TRL11/IRL11, UL119-118, m138, r138) (reviewed in (62).

2. EVASION OF THE ADAPTIVE IMMUNE RESPONSE

a. Effects of IL10

Cellular IL10 is a pleiotropic cytokine, with a variety of effector functions elicited depending on the cell type targeted. In the context of viral infection elevated systemic IL10 has been associated with the establishment and maintenance of chronic viral infection. While a cause and effect relationship has not been established between chronic CMV infection and IL10, a clear relationship between the two has been established for other persistent viruses including EBV, HCV and more convincingly LCMV (67). Altered cellular IL10 responses have been implicated in the maintenance of several chronic viral infections through the influence of IL10 on T helper cell lineage commitment, shifting the balance from a highly effective cell mediated T_H1 immune response to a lesser effective T_H2 humoral response (67).

Human Cytomegalovirus and Rhesus Cytomegalovirus have both been shown to encode functional IL10 homologues, while both Mouse and Human Cytomegalovirus have been demonstrated to upregulate cellular IL10 (147, 205, 213). The virally encoded cmvIL10 of HCMV and RhCMV were not immediately recognized in the initial annotation of the viral genomes, as they are only 27 and 25% homologous to their respective cellular IL10. Spencer and colleagues found that cmvIL10 possessed a number of anti-inflammatory properties including the

inhibition of mitogen stimulated PBMC proliferation, a decrease in proinflammatory (TNFα, GM-CSF, IL6, IL1a) cytokine production and a generalized inhibition of antigen presentation (257). IL10 effects on MHC I and MHC II down regulation will be discussed in greater detail in the CMV manipulation of antigen presentation section.

b. CMV Manipulation of Antigen Presentation

CMVs effectively down regulate multiple steps along the antigen presentation pathway resulting in the diminished presentation of viral antigen in the context of both MHC I and MHC II. Although the *in vivo* significance of CMV down regulation of MHC is still the subject of great debate, the phenomena of MHC down regulation has now been widely described for a number of CMVs, including HCMV, MCMV, RhCMV, and now RCMV. Recognition of foreign antigen displayed on the cell surface by major histocompatability molecules triggers T cell activation and the subsequent adaptive immune response to viral infection (111). The MHC I molecules transport antigen derived from the cytosol to the cell surface for recognition by cytotoxic CD8T cells (111). Antigen derived from the vesicular compartment is displayed on the cell surface by MHC II molecules and recognized by CD4T cells, specialized cells commonly referred to as "helper" T cells which activate macrophages, B cells and CD8T cells (111).

Antigenic peptides derived from the cytosol are generated by proteasome degradation of ubiquitinated protein. The peptides are shuttled into the endoplasmic reticulum (ER) by the ER resident transmembrane protein, transporter associated with antigen processing (TAP) (15). TAP is a heterodimeric protein, composed of two subunits, TAP1 and TAP2, which together form an active transport pore through which processed peptides gain entry to the ER. Further degradation of ER resident peptides is prevented by their association with peptide binding chaperone proteins, such as the recently identified protein disulfide isomerase (PDI) (192, 255). Once in the ER, peptides bind to newly synthesized MHC I in association with β2-microglobulin. The addition of peptide stabilizes the trimeric complex. Peptide bound MHC I-β2 microglobulin complexes are then transported through the Golgi to the plasma membrane (15).

Several ORFs have been identified in HCMV, MCMV and RhCMV which encode proteins manipulating various stages of the MHC I antigen presentation pathway. These are commonly referred to as VIPRs (viral genes that inhibit antigen presentation to CD8T cells) and include US2, 3, 6, 11, m4, 6,152, Rh182, 184, 185 and 189 (190, 197). Gene products encoded in the US2-11 region of HCMV disrupt various stages of the MHC I pathway. US2 and 11 have been shown to dislocate newly synthesized MHC I heavy chains (305) from the ER and into the cytosol where they are subsequently degraded by the proteasome (118, 303, 304). Peptide loading of nascent MHC I molecules is blocked by US6 binding to

the ER luminal side of TAP, thus preventing peptide transport into the ER. US3 blocks egress of MHC I complexes from the ER through its interaction with tapasin, a chaperone of MHC which facilitates peptide loading (6, 84, 118, 138, 303, 304). Recently US3 was also found to inhibit peptide loading of MHC I through its interaction with PDI, an ER resident peptide chaperone (192). Heterologous expression of US3 resulted in a depletion of PDI (192).

Although the rodent CMVs do not encode positional homologues to the US genes mediating MHC I down regulation, MCMV appears to encode functional homologues (reviewed in (197). Three ORFs have been identified in the MCMV genome whose gene products mediate MHC I down regulation: m4, m6 and m152. The first to be identified, m152, although classified as an early gene, is expressed with immediate early kinetics; transcripts can be detected as early as two hours post infection *in vitro* fibroblast culture (197) Protein expression can be seen as early as three hours post infection, peaking three hours later, between five to six hours post infection (197). Even though direct protein:protein interaction has not been described for m152 and MHC I, the former effectively blocks MHC I maturation post peptide loading, thus preventing MHC I transit to the medial Golgi (51).

During the initial studies describing m152, an additional regulator of MHC I surface expression was identified, m6 (215). Expression of m6 follows early kinetics of the CMV gene expression cascade, however high levels of the

encoded protein are maintained throughout the course of fibroblast infection. Through a direct interaction, m6 was found to reroute the mature trimeric MHC I from the Golgi through the endocytic pathway to the lysosome, where both MHC I and m6 are degraded.

The third ORF in MCMV found to interfere with CD8T cell recognition of infected fibroblasts, m4, was identified in a co-immunoprecipitation with MHC I as a binding partner of cellular MHC I (126). Unlike the previously described CMV gene products regulating MHC I surface expression, m4 neither mislocalizes nor causes the degradation of MHC I. Rather, m4 tightly binds to MHC I, escorting the mature trimeric complex to the plasma membrane where it remains associated with MHC I. The precise mechanism, through which, m4 blocks CTL recognition is still unknown.

Studies developing the RhCMV model system have uncovered additional functional homologues to the HCMV US6 gene family (190). ORFs Rh182, Rh184, 185, and 189 encode both positional and functional US6 family homologues (92, 190). Much like their counterparts in HCMV (US2 and US11), RhCMV ORFs Rh182 and 189 were shown to target the heavy chain of MHC I for proteasome mediated degradation. Using an Adenoviral vector to express either Rh182 or 189 in U373-MG cells, Pande *et al.* observed a rapid degradation of heavy chain and a dependence on proteasome inhibitors for the stabilization of nascent heavy chain (190). In the same study, transient expression of Rh185

was shown to inhibit peptide transport by TAP, a clear indication that Rh185 acts as a functional homologue of HCMV encoded US6 (190). Although Rh182, 185 and 189 have all been demonstrated to *independently* downregulate *surface levels* of MHC I, one other member of the US6 gene family is able to interfere with the MHC I pathway (190). Transient expression of Rh184 (homologue to US3) delays MHC I maturation in transiently transfected HeLa cells (190). While expression of Rh184 alone has no net affect on surface expression of MHC I, it is still unclear what contribution Rh184 may play in the context of infection. It is possible that during the course of a natural infection, delay of MHC I maturation may be critical to mediate efficient control by one or more other viral gene products.

More recent work examining RhCMV mediated down regulation of MHC I found that the deletion of the US2-11 region did not rescue MHC I in infected fibroblasts (203). A series of deletion mutants identified rh178 as the ORF responsible for MHC I depletion in the ΔUS2-11 RhCMV. Proteasome inhibition was not able to stabilize MHC I protein levels and expression of rh178 alone resulted in a block in MHC I heavy chain expression. Further investigation into the mechanism of rh178 down regulation of MHC I revealed wild type levels of heavy chain mRNA suggesting the loss of MHC I to be a post-transcriptional event, likely an interference in translation although polyribosome formation was not affected. Translation of MHC I heavy chain, a type I transmembrane protein, requires translocation across the ER membrane, a process mediated by an N-terminal signal peptide. Powers *et al.*, went on to confirm that rh178 mediated

interference with MHC I protein synthesis was dependent on the signal peptide and exchange of the MHC I signal sequence for that of CD4 or murine heavy chain was able to stabilize MHC I protein levels.

RCMV interference with MHC I cell surface expression was reported by Hassink *et al.*, as a transient event, with maximal down regulation (60% depletion) occurring twelve hours post infection in both primary and transformed fibroblasts (97). To date, this is the only published report of RCMV down regulation of antigen presentation. Reduced cell surface expression was caused by a delay in MHC I maturation in infected cells. It is not clear if the transient nature of the down regulation as compared to our findings in bone marrow derived dendritic cells (BMDC) is due to RCMV strain differences, or simply a cell type specific event. To date no RCMV ORF has been linked with MHC I or II down regulation.

MHC II Antigen Presentation Pathway

Mature MHC II molecules are heterotrimeric single pass transmembrane proteins consisting of one α chain, one β chain and bound peptide (reviewed in (44). Assembly of the mature complex begins in the ER where nascent α and β chain heterodimers complex with trimeric Invariant chain (Ii) (23, 71, 110) [FIGURE 4]. It is the binding of Ii chain to the newly synthesized $\alpha\beta$ chain dimers that prevents endogenous peptides shuttled into the ER (by TAP) from occupying the peptide-

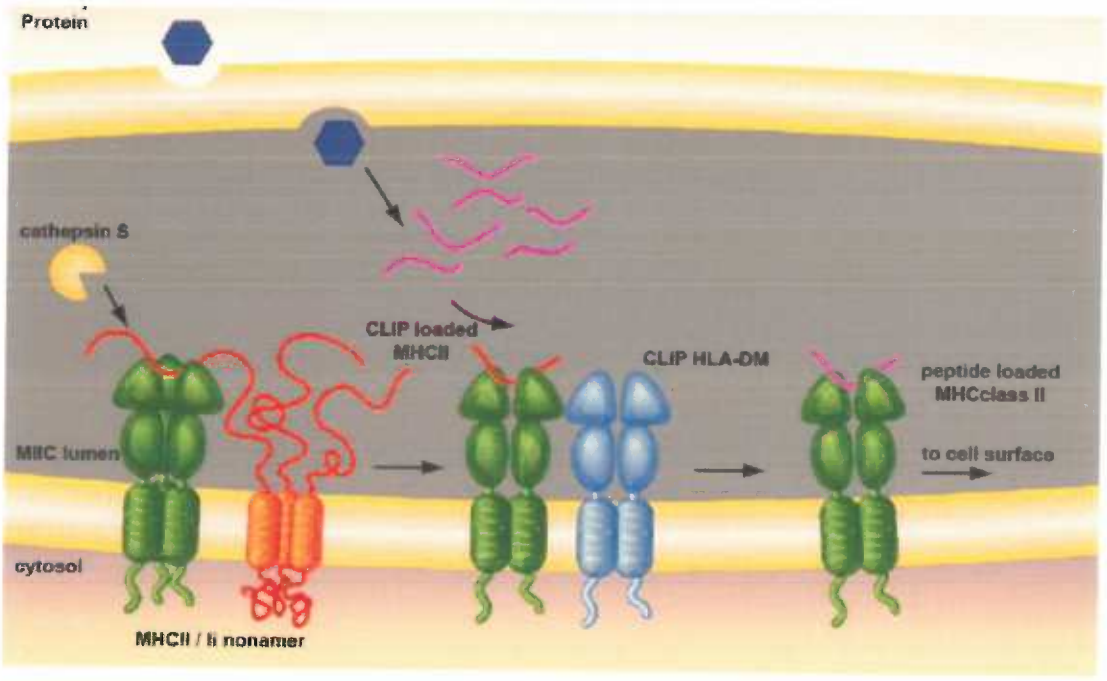

Figure 4. MHC II Classical Peptide Loading

Newly synthesized MHC IIαβ heterodimers (green) associate in a stepwise fashion to preformed Invariant chain trimeric complexes (red) in the ER. Formation of the nonameric complex signals the transit out of the ER via the Golgi to the MHC II Loading Compartment (MIIC) where acid activated proteases, such as Cathepsin S (yellow PacMan), digest the Ii chain to a small peptide called CLIP which resides in the peptide binding groove. Exchange of CLIP for antigenic peptide (pink) is catalyzed by HLA-DM, which also functions to stabilize MHC IIαβ heterodimers in the MIIC. Once loaded with peptide, mature MHC IIαβ is stable and transported to the plasma membrane.

Image courtesy of Andrew Townsend

binding groove (190). The stepwise addition of three αβ chain dimers to an Ii trimer, forms a nonameric complex which is then transported from the ER via the Golgi apparatus to specialized acidic vesicles called MHC II compartments (MIIC) (168). During transit from the ER to the MIIC, most likely in the late endosome, Ii chain is degraded, leaving behind a 23 amino acid residue peptide, termed CLIP, in the peptide binding groove (155). The exchange of CLIP for antigenic peptides is catalyzed by the enzymatic action of HLA-DM in the MIIC (41, 261). Antigenic peptides encountered by MHC II molecules in the MIIC are normally derived from either pathogens replicating in intracellular vesicles or extracellular proteins taken up via endocytosis or phagocytosis and subsequently degraded in the lysosomes by resident acid activated proteases, known as cathepsins. Peptides ranging from 13-25 amino acid residues in length are optimally exchanged for CLIP. Once peptide has been loaded, the mature MHC II molecule is then transferred to the plasma membrane where antigen is presented to CD4T cells.

HCMV inhibition of MHC II Antigen Presentation Pathway

Four ORFs in HCMV have been identified as MHC II down regulators: US2, 3, UL111A and UL83 (pp65) (115, 302). The first to be identified, US2, is an immediate early gene product (118) which mediates the proteasome dependent degradation of HLA-DMα and HLA-DRα (282). Although US2 mediated degradation of *class I* had been established, the finding that US2 caused the degradation of *class II* was somewhat surprising due to the limited sequence

similarity between HLA-A and HLA-DRα and –DMα (<27%) (116, 199). The degradation of DMα, DRα and HLA-A, but not β2m or DRβ-suggests that it is the membrane distal α1 domains (shared by DMα, DRα & β2m) and not the Ig domains (shared by all) that are targeted by US2 or US2 binding partners.

Due to the inherent difficulty of infection of cells naturally expressing high levels of MHC II, in their 1999 study Tomazin *et al.* employed a transfection based system to study the mechanism of US2 mediated down regulation of MHC II (281). A transformed astroglioma cell line, U373-MG, permissive for the complete replication cycle of the lab adapted HCMV strain, AD169, and IFNγ inducible for MHC II expression, was stably transfected with the MHC II transactivator (CIITA) to boost the constitutive level of MHC II expression in these cells (U373-CIITAHis).

The pilot experiments utilized the U373-MG cell line under IFNγ stimulation to induce low level endogenous MHC II expression (281). Cells, which were IFNγ stimulated at the time of infection with wild type AD169, had a marked reduction of newly synthesized HLA-DRα 12 hours post infection. Whereas in cells infected with an AD169 US2-11 knock out virus nascent HLA-DRα was found to be stable. In an effort to eliminate the possible inhibitory effects of CMV on IFNγ stimulation of MHC II via the Jak/Stat pathway, the U373-CIITAHis cell line was used in the remainder of the experiments to test CMV manipulation of constitutive MHC II

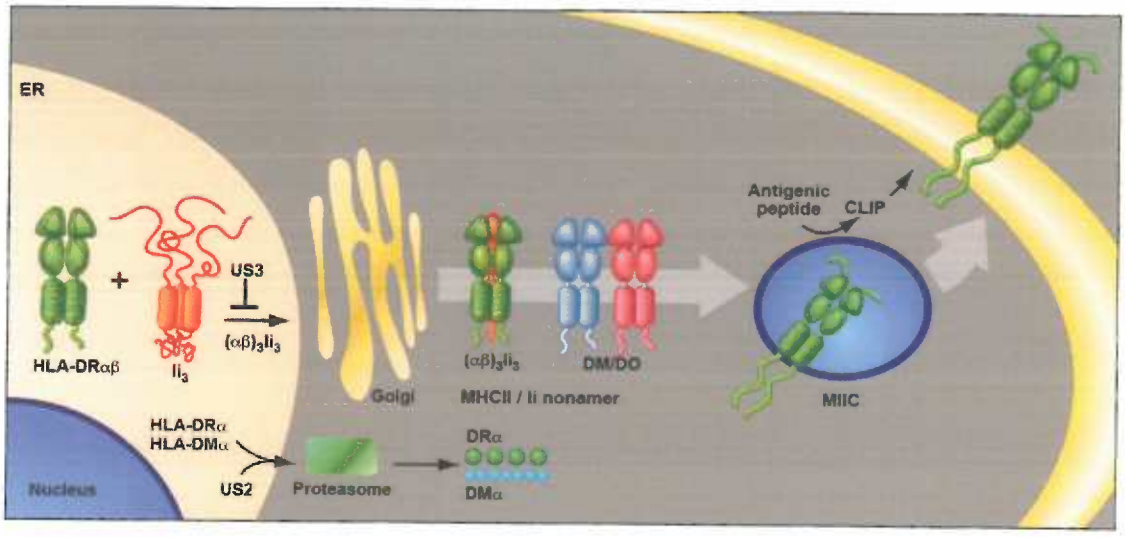

Figure 5. Cytomegalovirus Inhibition of MHC II Antigen Presentation Pathway

HLA-DRα/β heterodimers assemble onto preformed Invariant chain (Ii) homotrimers in a stepwise fashion in the endoplasmic reticulum (ER) until a nonameric complex is formed. Transit out of the ER through the trans golgi network is facilitated by HLA-DM and HLA-DO. Transit to the MIIC loading compartment results in the degradation of the Ii chain to a peptide fragment termed CLIP within the peptide binding groove of HLA-DRα/β. HLA-DM catalyzes the exchange of CLIP for the antigenic peptide and mature peptide loaded MHC II then transits to the plasma membrane. HCMV has been demonstrated to interfere with several steps of the pathway. HCMV encoded US3 blocks the assembly of HLA-DRα/β heterodimers onto the Ii chain trimer, thus blocking HLA-DR exit from the nucleus. US2 is able to directly bind HLA-DRα and has been shown to mediate the proteasome dependent degradation of both HLA-DMα and HLA-DRα.

Image courtesy of Andrew Townsend

expression (167). Infection of these cells with a panel of AD169 deletion mutants within the US2-11 region identified US2 as mediating the effect.

Tomazin and colleagues found that US2 is able to directly bind free HLA-DRα chain and likely complexed αβIi trimers. Exogenous expression of US2, using an adenoviral expression system found that both HLA-DRα and HLA-DMα are degraded in cells expressing US2. Furthermore, degradation of HLA-DRα was found to be proteasome mediated. US2 expression in both the U373 cell lines was found to inhibit the presentation of exogenous antigen to CD4T cells.

The next ORF identified in HCMV interference with the MHC II antigen presentation pathway, US3, was discovered utilizing the same MIITC transfected astroglioma model system employed to explore the mechanism of US2 effect on HLA-DRα and –DMα (100). Hegde *et al.* found that exogenous expression of US3 inhibits HLA-DR assembly, transport and peptide loading, culminating in a functional defect in MHC II antigen presentation to CD4T cells. Immunoprecipitation experiments performed on metabolically labeled cells demonstrated that US3 binds HLA-DRα/β heterodimers in the ER. Furthermore, a three- to four-fold reduction in the amount of Ii chain associated with the α/β heterodimers immunoprecipitated with US3, suggests that US3 binding inhibits Ii chain association with HLA-DR. Subsequent transit to the Golgi was not affected by US3 expression, although subcellular fractionation of metabolically labeled cells revealed a depletion of nascent α/β heterodimers in the more dense

lysosomal fractions. Additional evidence suggesting that HLA-DRα/β heterodimers do not complete their transit to the MIITC loading compartment is the observed three to nine-fold reduction in SDS stable peptide loaded dimers.

In 2003 the Söderberg-Nauclér group reported an additional HCMV encoded protein involved in manipulation of the MHC II antigen presentation (184). Odeberg, *et al.* identified UL83 as a viral ORF involved in the early phase of CMV induced MHC II down regulation. The initial characterization of HCMV induced MHC II depletion was carried out in IFNγ treated fibroblasts (with induced MHC II expression) infected at high multiplicity with AD169, Towne, HA (a clinical isolate), and two deletion mutants UL18ΔHCMV and RVAD65 (a UL83 deletion mutant). Infection of IFNγ treated fibroblasts with the RVAD65 mutant resulted in "wild type" levels of MHC II at 24 hours post infection. Additionally, transient transfection with pCDNA3 containing the UL83 ORF, demonstrated that pp65 is sufficient to down regulate MHC II in their induced fibroblast culture model.

In an effort to confirm the effect in a relevant cell type, Odeberg *et al.* examined HLA-DR surface levels in PBMC-derived *in vitro* differentiated DCs 24 hours post infection (MOI equal to 25-40) with both AD169 and RVAD65 (184). On average, infection efficiencies (as monitored by viral pp52 staining) ranged from 1-5%, with the highest level of infection at 15-20% in two donors. HLA-DR surface levels in the more susceptible donors' DCs were reduced by 50% 24hpi with wild type

AD169 whereas only a slight reduction (relative to uninfected PBDC) in HLA-DR was seen in RVAD65 infected DCs.

Further biochemical characterization of the effect of pp65 on the MHC II antigen presentation pathway was carried out in the induced fibroblast system, due to the extremely inefficient infection of PBDCs with the lab adapted strain, AD169 (184). RT-PCR analysis revealed no effect on HLA-DRα transcription. Rather a loss of newly synthesized as well as steady state levels of HLA-DRα, with no effect on HLA-DRβ, -DM or Ii levels, was observed. Odeberg and colleagues attributed the loss of nascent HLA-DR complexes to accumulation in perinuclear lysosomes (as seen in IFA) followed by subsequent degradation within this compartment although the link between the enhanced lysosomal accumulation and degradation was never clearly established. Furthermore, pp65 was shown to localize to the nucleus (not the lysosome) and a precise role for pp65 in MHC II degradation has yet to be elucidated.

Effects of CMV Induced/Encoded IL-10 on MHC II

The first published report describing the effects of CMV induced IL10 on MHC II surface expression utilized the murine CMV model (213). Cell surface levels of MHC II were reduced by less than 50% in macrophages infected with MCMV at high multiplicity (MOI equal to 10). The effect on MHC II was not mediated by viral binding, entry or a structural component, but rather required viral gene

expression, as UV-inactivated virus did not result in a reduction of surface expression. Additionally, the transfer of virus-free cultured supernatant taken from infected cells was able to deplete MHC II in uninfected macrophages equivalent to levels observed in macrophages infected with MCMV. The ORF(s) responsible for MCMV-induced depletion of MHC II have yet to be identified.

While the effects of CMV-induced cellular IL10 on MHC II surface expression in infected macrophages were first described in 1999, it was not until 2002 that a viral ORF (the RhCMV homologue to HCMV UL111A), encoding an IL10 homolog, was recognized as a modulator of the MHC II antigen presentation pathway (257). Spencer *et al.* published the first functional analysis of the viral IL10, cmvIL10 (257). Treatment of monocytes with cultured supernatant taken from RhCMV UL111A transfected HEK cells, resulted in a nearly three fold reduction in HLA-DR surface expression. In 2004 two groups working independently published their findings on the effects of recombinant HCMV UL111A and cultured supernatants (derived from infected fibroblasts) to modulate PBDC function, including MHC II surface expression (38, 206). Again, due to the inefficient infection of PBDC with lab-adapted strains of HCMV, the studies were both performed out of the context of viral infection. Treatment of immature DCs with recombinant cmvIL10 resulted in a 30% reduction in MHC II cell surface MFI (206).

In addition to the known ORFs mediating the down regulation of MHC II, both HCMV and MCMV have been shown to inhibit induced upregulation of MHC II expression by unknown mechanisms. Sedmak and colleagues showed that induction of IFNβ in HCMV infected ECs block IFNγ induced upregulation of MHC II (234). Similarly MCMV was shown to inhibit MHC II expression on macrophages via induction of IFNβ (101).

D. HYPOTHESIS

While antigen presenting cells are believed to play a critical role in the lifecycle of Cytomegaloviruses *in vivo*, little is known about the dynamics of infection of APCs *in vivo*, what the cellular requirements for infection are, which viral gene products mediate long term maintenance of the virus *in vivo* and whether infection of APCs is a requirement for persistent infection. The studies outlined in the subsequent chapters center on understanding the cellular and viral parameters to the establishment of CMV *in vivo*, specifically with regard to the impact of infection of APCs on both APC function and the viral life cycle and ultimately the establishment of a persistent or latent infection.

We know from the literature that both Human Cytomegalovirus and Mouse Cytomegalovirus down regulate MHC II from the surface of infected macrophages and dendritic cells. Four ORFs whose products mediate MHC II depletion have been identified in HCMV: US2, US3, UL111A and UL83. While a

viral gene product mediating MHC II depletion in the context of MCMV infection has not been identified, the induction of cellular IL10 has been clearly linked to MCMV induced depletion of MHC II. Likewise, HCMV has been demonstrated to induce cellular IL10 as well as encode a functional homologue of cellular IL10 (cmvIL10), both of which mediate the loss of cell surface MHC II in infected cultures. Furthermore, the induction of IL10 was found to have a paracrine effect on uninfected cells cultured with virus free conditioned supernatant (and recombinant cmvIL10 encoded by UL111A) and the down regulation of MHC II via cultured supernatant was eliminated with the use of neutralizing anti-IL10 antibody.

Initial studies conducted in the Streblow and Nelson laboratories investigating Rat CMV infection of bone marrow derived dendritic cells (BMDC), observed a striking depletion of MHC II in infected BMDC. Use of a recombinant Rat CMV expressing GFP from the cellular promoter, eFIα, facilitated the labeling of infected cells and subsequent investigation of the direct effects of infection versus indirect paracrine effects (*i.e.* IL10 and other secreted mediators). RCMV infection of BMDC was found to not mediate the down regulation of MHC II in neighboring uninfected (GFP negative) cells. Nor was UV-inactivated virus able to mediate the depletion of MHC II as previously observed in the studies identifying the UL89 gene product as an early mediator of MHC II depletion. These early findings coupled with the lack of sequence homologues to the US2, US3 and UL111A ORFs, lead to several questions. Does Rat CMV deplete cell

surface MHC II through proteasome mediated degradation of nascent MHC II alpha chain as US2 does? Does RCMV inhibit MHC II egress from the ER as US3 does? Does RCMV mediate the depletion of MHC II by the same mechanisms as observed in HCMV, but by unique viral gene products? Alternatively, does RCMV depletion of MHC II occur by a novel mechanism altogether?

The data presented in Chapter three describe the development of *in vivo* models to examine the parameters of CMV persistence and dissemination. A multitude of studies have implicated both the peripheral blood and bone marrow as sites of CMV persistence or latency *in vivo*. However, isolation of cell types infected in both reservoirs have been hampered by the low numbers of cells identified as infected and biased means of identifying those cells reliant on the expression of specific viral ORFs. The data presented by Streblow *et al.* detailing the widely divergent transcriptional profiles seen in tissues infected *in vivo* compared to infected fibroblast cultures highlights the need for an unbiased means of identifying and isolating cells infected *in vivo*. Chapter three details the use of the RCMV-GFP clone to identify and isolate cells of the peripheral blood and bone marrow infected *in vivo*. Identification and isolation of infected cells will permit future studies examining transcriptional profiles and cellular phenotype. Do different reservoirs of CMV express unique subsets of the viral genome, do these subsets change over the course of infection, are there transcriptional profiles unique to a persistent versus acute infection, does CMV encode genes

specifically associated with latent infection? A complementary approach to identification and isolation of infected cells to uncover both the cellular and viral factors contributing to persistence is the Cre-Lox approach to eliminate suspected reservoirs of virus *in vivo*. Viral mutants with floxed essential regions of the genome designed to be specifically inactivated in Cre expressing cells were used in combination with transgenic mice expressing the Cre recombinase under macrophage or endothelial cell specific promoters. This approach is designed to effectively eliminate viral replication in specific subsets of target cells *in vivo*, while maintaining the integrity of the viral genome and normal replication in cells not targeted for a replication knock out. Use of the Cre-Lox approach will facilitate future studies examining the cell type requirements for persistence, latency, pathogenesis and viral dissemination *in vivo*. These models are in the early stages of development and as such long term infections have not been examined, instead acute infection data is presented as the foundation for future studies examining persistence.

CHAPTER TWO: RAT CYTOMEGALOVIRUS INFECTION DEPLETES MHC I AND II IN BONE MARROW DERIVED DENDRITIC CELLS

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A version of this chapter is in review at *the Journal of. Virology*

ABSTRACT

While cytomegalovirus (CMV) infects and replicates in a multitude of cell types, the ability of the virus to replicate in professional antigen presenting cells (APCs) is believed to play a critical role in viral dissemination and latency. CMV infection of APCs and manipulation of their function has been an important area of investigation. CMV down regulation of MHC II is reportedly mediated by the HCMV proteins US2, US3, UL111a (vIL10) or through the induction of cellular IL10. In this study, we demonstrate that rat CMV (RCMV) significantly reduces MHC I and II expression by mechanisms that do not involve orthologues of the known HCMV genes nor by an increase in cellular IL10. Rat bone marrow derived dendritic cells (BMDC) were highly susceptible to infection with RCMV and a recombinant RCMV expressing eGFP. RCMV infection of BMDCs depleted both surface and intracellular MHC II to nearly undetectable levels as well as reduced surface expression of MHC I. The effect on MHC II only occurred in the infected GFP positive cells and is mediated by an immediate early or early viral gene product. Furthermore, treatment of uninfected iDCs with virus-free conditioned supernatants from infected cells failed to down regulate MHC II. RCMV depletion of MHCII was sensitve to treatment with lysosomal inhibitors but not proteasomal inhibitors suggesting that the mechanism of RCMV mediated down-regulation of MHCII occurs through endocytic degradation. Since RCMV does not encode homologues of US2, US3 or UL111a, these data indicate a different mechanism for RCMV depletion of MHC II.

INTRODUCTION

Cytomegaloviruses (CMV) are ubiquitous, species-specific β-herpesviruses. Between fifty and ninety percent of adults worldwide are infected with Human Cytomegalovirus (HCMV) (169). While infection with HCMV is normally asymptomatic in the immunocompetent host, immunosuppressed individuals and neonates infected with the virus may develop severe complications including pneumonia, retinitis, gastrointestinal disease and death. Primary HCMV infection typically results in life long persistence characterized by a latent phase with intermittent reactivations and secondary infections (169). The establishment of this persistent infection provides a unique challenge to the virus, which is under constant surveillance by the host immune system. CMVs derive their success in maintaining their prevalence from their ability to evade immune recognition and clearance through the acquisition of genes specifically targeting both the adaptive and innate immune response. With a coding capacity of approximately 180 predicted open reading frames, only 40-60 of which have been demonstrated to be essential for *in vitro* viral replication, it is estimated that nearly two thirds of the CMV genome is devoted to sustaining infection *in vivo*, a prime component of which is subversion of host immune surveillance (55, 171).

The adaptive strategies employed by CMV to counteract the host immune system fall into two broad categories: immunomodulation and immune evasion. Examples of immunomodulation can be found in viral recruitment of leukocytes to

the site of primary infection through the expression of virus-encoded chemokines and through virus-induced expression of host cell cytokines and growth factors. Dampening of the inflammatory response and virus-induced functional paralysis of antigen presenting cells (APCs) such as dendritic cells (DC) and macrophages occurs through the altered cytokine profiles of infected cells as well as by the down regulation of host cell receptors and ligands (171). While evasion strategies implemented by CMV include the ability of the virus to maintain a quiescent infection in APCs, the prevailing paradigm of immune evasion is typified by CMV manipulation of both the classical and nonclassical major histocompatability complexes.

The capacity of APCs to present antigen in the context of MHC I and II is central to the ability of the host to activate the adaptive immune response to viral infection. Like other members of the herpesvirus family, CMV has evolved to avoid host immune detection by manipulating MHC processing and presentation (reviewed in (143)). Several ORFs have been identified in both mouse CMV (MCMV) and HCMV that mediate MHC I down regulation at various stages of MHC processing. Three MCMV ORFs, m4, m6 and m152, encode glycoproteins with nonredundant and complementary function in MHC I interference (123). M4 binds MHC I in the ER and is transported to the cell surface where it interferes with CD8+ T cell recognition of MHC I (124, 126). M6 redirects assembled MHC I:peptide complex from the endoplasmic reticulum (ER) to the lysosome where it is subsequently degraded (215). M152 retains MHC I in the ER/Golgi

compartment, thus blocking plasma membrane localization (314, 315). The glycoproteins encoded by the four HCMV ORFs, US2, US3, US6 and US11 have all been associated with post translational blockade at several critical steps in MHC I processing (7). US2 and US11 inhibit cell surface expression of MHC I and US3 and US6 both block peptide loading by inhibiting tapasin and Transporter associated with Antigen Processing (TAP).

A critical component of the CMV life cycle is the ability of the virus to replicate in MHC II positive cells, including endothelial cells, epithelial cells, monocytes, macrophages and DCs (69, 146, 166). Infection of macrophage progenitors is believed to play a pivotal role in viral dissemination and reactivation from latent or low level persistent infections. While a variety of mechanisms and virus-encoded ORFs that mediate MHC I down regulation have been extensively studied, relatively little is known about CMV induced MHC II down regulation. Four HCMV ORFs have been identified as modulators of MHC II cell surface expression: US2, US3, UL83 and UL111A (vIL10) (38, 100, 281). US2 modulates MHC II cell surface expression by directly causing the degradation of HLA-DRa and destabilizes newly formed MHC II α/β complex (281). US3 blocks efficient sorting of MHC II molecules to the peptide loading compartment by binding class II α/β complexes, which compromises the stability of those complexes (100). In fibroblasts induced to express MHC II, UL83 was shown to down regulate cell surface HLA-DRα by an unknown mechanism (184). Studies investigating vIL10 have demonstrated that it is both a structural and functional

homologue of cellular IL10, however there are conflicting reports on the effectiveness of the viral cytokine to down regulate cell surface MHC II (132). For example, Raftery *et al.* have shown that vIL10 blocked LPS induced MHC II cell surface expression in immature peripheral blood derived dendritic cells (PBDC); while Chang *et al.* reported that vIL10 has no effect on MHC II expression in mature PBDC (38, 206). MCMV lacks an IL10 homologue, however, the virus down regulates surface MHC II on primary macrophages and the macrophage cell line IC21 by inducing secretion of cellular IL10 (213).

Several laboratories have outlined the impact of direct infection of APCs on their ability to initiate both innate and adaptive immune responses to CMV *in vitro*. Likewise, human DC function in the context of a natural HCMV infection has also been analyzed (73, 289, 290). Circulating DC obtained from patients undergoing active CMV infection were both phenotypically and functionally impaired with a 50% reduction in MHC II (73). Furthermore, DC derived from heart transplant recipients with acute CMV disease have reduced MHC II and diminished ability to stimulate T cell proliferation (289). The current study utilizes a direct infectivity model of rat bone marrow derived DC (BMDC) to investigate RCMV induced MHC manipulation. DC progenitors are highly abundant in the bone marrow, easily accessible and an important cell type in CMV biology. While RCMV has previously been shown to transiently down regulate MHC I in fibroblasts, there are no published reports describing RCMV infection of DC or the ability of the virus to manipulate MHC II. Here we demonstrate that immature rat DCs are

highly susceptible to RCMV infection, and that infection of these cells results in a greater than 90% reduction in the levels of intracellular and cell surface MHC II. The virus-induced depletion of MHC II is the result of direct infection, requiring immediate early and/or early viral gene expression, is not mediated by a secreted factor and appears to occur by at least two mechanisms: one affecting synthesis of nascent MHC II alpha, the other degrading mature MHC II by acid dependent proteases.

MATERIALS & METHODS

Generation of Bone Marrow Derived DCs

Rodents were housed in the AAALAC accredited Portland VA Medical Center animal facility in a specific-pathogen-free room in compliance with USDA/HHS guidelines. Bone marrow was flushed from the femurs and tibias of Lewis and F344 adult male rats and strained through a 70μm filter. Bone marrow cells were washed twice in complete RPMI-1640 medium supplemented with 10% v/v fetal bovine serum, 100 U/ml penicillin, 100μg/ml streptomycin and 2mM L-glutamine. Bone marrow cells were then cultured at a cell density of $1x10^6$ cells per ml in complete RPMI supplemented with 10ng/ml recombinant rat GM-CSF (R&D Systems, 518-GM) and 5ng/ml recombinant rat IL4 (R&D Systems), in a final volume of 10ml. Five days after plating, the cells were given fresh media plus cytokine. Nine days after initial plating, the non-adherent cells were removed from the culture; adherent cells were washed three times with DPBS, once with RPMI and then cultured in complete RPMI without cytokine. In order to stimulate the immature BMDCs to mature, the cells were treated with 1μg/ml LPS. After an additional 24-hour incubation, the LPS-matured or immature BMDC were washed twice with DPBS and once with RPMI, and refed with complete RPMI.

Rat Cytomegalovirus

Tissue culture-derived stocks of the Maastricht strain of RCMV were amplified and titered in rat lung fibroblasts (RFL6s). Cell free virus was obtained from the

cultured supernatants of infected cells, which were spun at 2k RPM for 20 min to remove cell debris, and then pelleted at 22k RPM (Beckman SW28) through a sorbitol cushion (20% D-sorbitol, 0.05M Tris, 1mM $MgCl_2$). The virus pellet was resuspended in Minimal Essential Medium (MEM) culture media. Plaque assays were performed in confluent 24-well plates by infection with an appropriate serial virus dilution in 0.2 ml of media and then incubated at 37°C for three hours. Following incubation, the infected cells were overlaid with 1 ml MEM supplemented with 10% fetal bovine serum (FBS), non-essential amino acids, pen/strep and 20 mM L-Glutamine with carboxymethylcellulose (Sigma, St. Louis, MO). After 7 days, the cells were fixed in 3.7% formalin in PBS and stained with 0.05% aqueous methylene blue (Sigma). The plaques were counted by light microscopy. To inactivate RCMV replication, the required PFU of infectious virus was diluted in serum-free medium in 10cm dish and exposed for 4 min at 999 $\mu J/cm^2$ in a UV-Crosslinker (Fisherbrand-UVXL-1000), which we have previously determined is sufficient to inactivate 100% of RCMV replication in fibroblasts using immunostaining at 24hrs with antibody to IE gene product. In addition, the RCMV DNA polymerase inhibitor, Foscarnet (0.5mM) was used to treat infected cell cultures at 8 hours post infection in order to determine whether the effect of the RCMV down regulation of MHC was due to the expression of a late viral gene product.

Construction of RCMV-GFP

To mark infected cells, we constructed a recombinant RCMV strain (RCMV-Δ145-7GFP), in which the ORFs r145, r146 and part of r147 were deleted and replaced by homologous recombination with a GFP expression cassette. The recombination plasmid pΔR145-7 was constructed as follows. A 1kb region of RCMV from 196,976 to 197,998 (RB1) was produced by PCR with the primers r144Fwd: ata aag ctt atc gcg gac gcg gac agc gag ata t and r144Rev: ata gat ctt taa tta acg gga ttg aga tat acg tac acc gtg and cloned into pCDNA-3 using the restriction enzymes HindIII and NotI. The primers for the second PCR fragment from 200,771 to 201,803 were r147Fwd: ata tta att aat gta tca ggc acc gtg tac tcg ata acg and r147Rev: ata gcg gcc gca agc ttg agg tag aag tag aat aaa gcg tta tga. The second fragment (RB2) was cloned into pCDNA-3Δr144 using NotI and XhoI. The GFP cassette was blunt end inserted into the Not1 site found in pCDNA-3Δr144-7. The EF-1α/eGFP cassette from the vector pQ100 (a gift from Dr. J. Vieira, University of Washington) was cut with EcoRV/XhoI and blunt ended for subsequent cloning into the recombination vector to make pΔR145-7. Since the primer r147 Rev also contained an internal HindIII site, this enzyme was used to release a 3.5kb fragment containing the RCMV flanking regions and GFP cassette.

The virus was constructed by transfecting HindIII cut pΔR145-7 into $1x10^6$ RFL-6 cells (260kV, 975μFarrads) with a GenePulser (BioRad). The cells were plated in complete DMEM for 6 hours and then infected with $1x10^5$ PFU of salivary gland-derived RCMV. After two days the cells were scraped, and cells and

supernatants were frozen at -80°C. The mix was thawed and added to fresh RFL-6 cells. On the second pass, GFP+ plaques were isolated and subcloned by limiting dilution on RFL-6 cells in 96-well plates (4 times). The integrity and clonal purity of the recombinant virus were confirmed by restriction endonuclease digestions in combination with Southern blot analysis (data not shown).

Antibodies

The following primary antibodies were used for phenotypic analysis of BMDCs and splenocytes: MHC II [RT1.B] (Serotec, MRC OX-6), MHC II [RT1.D] (Serotec, OX-17), MHC I [RT1.A] (Serotec, OX-18), CD80 (Serotec, 3H5), CD86 (Serotec, MRC OX-48), CD44H (BD Pharmingen, OX-49), CD4 (BD Pharmingen, OX-38), CD8α (BD Pharmingen, OX-8), CD11b (Serotec, OX-42), CD11c (Serotec, 8A2), CD45R (BD Pharmingen, HIS24), CD172a (Serotec, OX-41), OX-62 (Serotec, MRC OX-62), IL10 (PeproTech Inc., 500-P139), RhoA (Santa Cruz, sc-179) and GAPDH (AbCAM, ab8245). The following secondary antibodies were used: anti-mouse IgG APC (eBiosciences, 17401282), donkey anti-rabbit HRP (Amersham, NA934V), swine anti-goat HRP (Biosource, ACI 3404), anti-mouse HRP (Amersham, NXA-931). Isotype Controls: IgG2a (BD Pharmingen, 553454), IgG1 (BD Pharmingen, 550615), IgG1 (BD Pharmingen, 557273).

Flow Cytometry

BMDCs were removed from plastic dishes with gentle scraping, and spun at 2k RPM for 5min to pellet. The pelleted cells were washed in DPBS one time, resuspended at a cell density of $1x10^7$ cells per ml in FACS Block Buffer (20% Normal Goat Serum in Dulbecco's Phosphate Buffered Saline, 0.1% NaN_3) and incubated on ice for 20 min. Blocked cells were spun at 2k RPM for 5 min and resuspended in FACS Wash Buffer (1% NGS in DPBS, 0.01% NaN_3) at $1x10^7$ cells per ml. A total of $1x10^6$ cells were stained with each appropriate primary antibody for 30 min on ice. Following primary antibody incubation, cells were washed three times with FACS Wash Buffer and resuspended in 200µl FACS Wash Buffer and incubated with the appropriate secondary antibody or streptavidin-allophycocyanin (BD Pharmingen, 554067) for 30 min on ice. The stained cells were washed twice with FACS Wash Buffer and analyzed on a FACS Calibur (Becton Dickenson) utilizing Cell Quest software. Final data analysis was performed using FlowJo software (Tree Star, Inc.).

Immunofluorescence Microscopy

BMDC samples were cytospun (Shandon) onto glass slides, which were subsequently washed with PBS and fixed with 2% PFA in PBS. For intracellular staining, the samples were permeabilized and blocked in intracellular staining buffer (ISB: 1g BSA, sodium azide, 0.5% Triton-X100, and 500ml PBS) with 10% normal goat serum (NGS) for 20 minutes. The primary antibodies (diluted in ISB) directed against MHC II, CD44H, and RCMV IE were incubated for 2 hrs at room

temperature. IgG isotype antibodies were used as a negative staining control. Samples were washed with ISB and incubated with fluorescein isothiocyanate (FITC) or L-rhodamine conjugated secondary anti-mouse or anti-rabbit antibodies (BioSource International, Camarillo, CA), diluted in ISB for 1hr. Samples were washed with ISB, mounted and visualized using a Delta Vision RT microscope by Applied Precision. Photomicrographs were obtained at 60x magnification.

Western Blot

Total cell lysates from mock infected iDC or iDC infected at an MOI=1 treated with and without epoximicin or bafilomycin were run on 12% polyacrylamide SDS PAGE gels. Proteins were transferred to PVDF membrane (Millipore). The blots were blocked with 5% milk in DPBS, and then incubated with the indicated primary antibody. Membranes were washed with Tris Buffered Saline plus 0.2% Tween 20 (TBS-T), incubated with the corresponding HRP conjugated secondary antibody, and washed with TBS-T. The proteins were visualized with the Advance ECL Western Blot Detection Kit (Amersham, RPN2135) and autoradiography. Protein levels were normalized to GAPDH or RhoA.

Mixed Leukocyte Reaction

Lewis rat BMDCs were prepared as described above and mock-infected or infected at an MOI equal to 1.0 with WT RCMV. Allogeneic splenocytes were prepared from macerated whole spleens harvested from ACI strain rats, washed three times in sterile DPBS and stained with CFSE (CellTrace CFSE Cell

Proliferation Kit, Molecular Probes) per manufacturer's instructions. Labeled splenocytes were cocultured with mock- or RCMV-infected BMDCs (72hpi) at a 1:1 ratio for five days and then stained for CD4. T cell proliferation was measured as a function of CFSE dilution in the CD4+ cell population by flow cytometry.

Metabolic Labeling, Proteasome Inhibition

BMDCs were gently scraped from the plates at 24hpi, washed twice in DPBS and incubated for 1hr at 37°C in starvation media (Hepes buffered RPMI without cysteine or methionine). Following starvation, cells were pulsed with ^{35}S-methionine/cysteine (Perkin Elmer, Easy Tag ^{35}S protein labeling mix) at 0.2mCi/ml for 20 min. After the pulse, cells were diluted in chase media containing an excess of cold cysteine and methionine and then washed two times with ice cold PBS. Cells were then chased for the indicated times at 37°C. Samples treated with inhibitor were incubated with either 0.1μM epoximicin (Peptides International, IEP-4381-V) or 50μM lactacystin (Sigma, L6785) throughout the starvation, pulse, and chase periods.

Immunoprecipitation

Cell pellets were lysed on ice in lysis buffer (0.5% NP-40, 50mM Tris HCl pH 7.4, 5mM $MgCl_2$, 1mM PMSF, 150mM NaCl plus protease inhibitors). Lysates were preabsorbed with Protein A/G agarose beads preincubated with 2μg IgG isotype control Ab for 30 min at 4°C. After the 30 min incubation the bead/lysate mixture

was spun at 10k RPM for 5 min and the cleared supernatant transferred to fresh Eppendorf tube containing 2µg of the indicated antibody and rotated for 1hr at 4°C, followed by a 2hr incubation with Protein A/G beads. Beads were washed four times with PBS, pelleted and resuspended in Laemelli's buffer and analyzed by SDS-PAGE and visualized by autoradiography.

Quantitative RT-PCR detection of MHC I and MHC II

Total RNA was prepared from uninfected and infected BMDC using the Trizol method (Gibco BRL) at 12, 24 and 36 hpi. cDNA was generated using Superscript III RT (Invitrogen) and analyzed by real-time PCR techniques using primer sets recognizing RT1A (fwd: ACAGATCACCCGGAACAAGTG, rev: ATCTGCGGAGCGACTCCAC), RT1B (fwd: ACAACCTGCTGGTCTGCTCA, rev: TCCCCGTTCCTAATAAGCTGTG), and L32 (fwd: GAAGATTCAAGGGCCAGATCC, rev: GTGGACCAGAAACTTCCGGA). Primers sets were identified using Primer Express software (Applied Biosystems). RT-PCR reactions were performed using the SYBR Green PCR Master Mix (Applied Biosystems). Following thermal activation of AmpliTaq Gold (10 min. at 95°C), a total of 40 cycles were performed (15 sec. at 95°C and 1 min. at 58°C) using the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Plasmid clones containing each gene fragment were used as positive controls and quantification standards. PCR results were analyzed using ABI Prism 7700 Sequence Detection Software. The sensitivity of detection of this assay was <100 plasmid copies for the tested genes. The ribosomal subunit, L32, was used to

normalize the expression data. Quantitative PCR data were analyzed by ANOVA and student's t-test.

Viability Assay

BMDCs were fixed in Fixation Buffer (BioLegend) at 24hpi post infection and washed in Permeabilization Buffer (eBioscience). BMDCs permeabilized with 1% paraformaldehyde served as non-viable positive controls. Cells were scraped and washed two times in cold PBS and then resuspended in FACS Wash Buffer at 1-2x10^6 cells per ml to which 5µl of a 7-amino-actinomycin D (7-AAD) Viability Staining Solution (eBioscience) was added. Cells were incubated with the 7-AAD solution for 20 minutes at 4C in the dark.

1. RESULTS

Rat bone marrow derived dendritic cells are highly susceptible to RCMV infection.

Dendritic cells and macrophages play an important role in immune surveillance and CMV dissemination throughout the infected host, as well as development and maintenance of latency and reactivation. Previous reports utilizing peripheral blood derived DC and DC/APC cell lines have demonstrated a limited susceptibility of *in vitro* cultured murine and human DC to MCMV and HCMV infections, respectively. Currently there are no published reports demonstrating Rat CMV (RCMV) infection of rat DCs. Therefore, we developed an *in vitro* model using RCMV infected BMDCs to examine the effects of RCMV on DC immunobiology. In order to derive dendritic cell cultures, bone marrow cells were cultured for 9 days in RPMI containing 10% fetal bovine serum, GM-CSF (10ng/ml) and IL4 (5ng/ml) (179). Fresh media with cytokine was added on day 5-post plating. At 9 days, the nonadherent cells were removed from the culture through washing, leaving only those cells that were tightly adherent. The adherent BMDCs were fed fresh media without cytokine and incubated for 24 hours (hrs) in the presence or absence of LPS.

BMDCs were analyzed by flow cytometry for cell surface markers, including CD4, CD8α, CD11b, CD11c, CD45R-B220, CD86 (B7.2), CD80 (B7.1), CD172a, *OX42*, *OX62*, and major histocompatibility marker complexes, MHC I (RT1A),

MHC II (RT1B) and MHC II (RT1D). BMC cultured under the conditions described above express classical dendritic cell markers, including inherently high levels of MHC I and II (Figure 6). BMDC cultured in the absence of LPS were defined as immature (iDC) due to their low levels of costimulatory molecules, CD80 and CD86. BMC incubated in the presence of LPS for 24 hrs had increased cell surface expression of CD86, CD80, MHC I and MHC II relative to mock treated BMC (data not shown), which is a characteristic of DC maturation (179).

BMDCs were infected with either wild type RCMV (WT) or a recombinant RCMV expressing GFP under an eF1α promoter in order to mark the infected DC and compare the direct and indirect effects of viral infection in infected versus uninfected cells from the same culture. Immature and mature (LPS treated) BMDC were mock-infected or infected with RCMV-WT or RCMV-GFP at a multiplicity of infection (MOI) equal to 0.5 or 1.0. Infection was assessed by flow cytometry for GFP expression 48hrs post infection (hpi) (Figure 7A). Uninfected cells (mock) and those cells infected with a non-GFP expressing RCMV (RCMV-WT) served as negative controls for autofluorescence. We found that rat BMDCs are highly susceptible to RCMV infection. Greater than 90% of iDC were GFP positive when infected at an MOI of 1.0 with RCMV-GFP. Figure 7B demonstrates that all of the GFP positive cells also stained positive by immunofluorescence for RCMV-Immediate Early proteins. In general, the infected cells displayed uniformly high levels (mean fluorescent intensities; MFI)

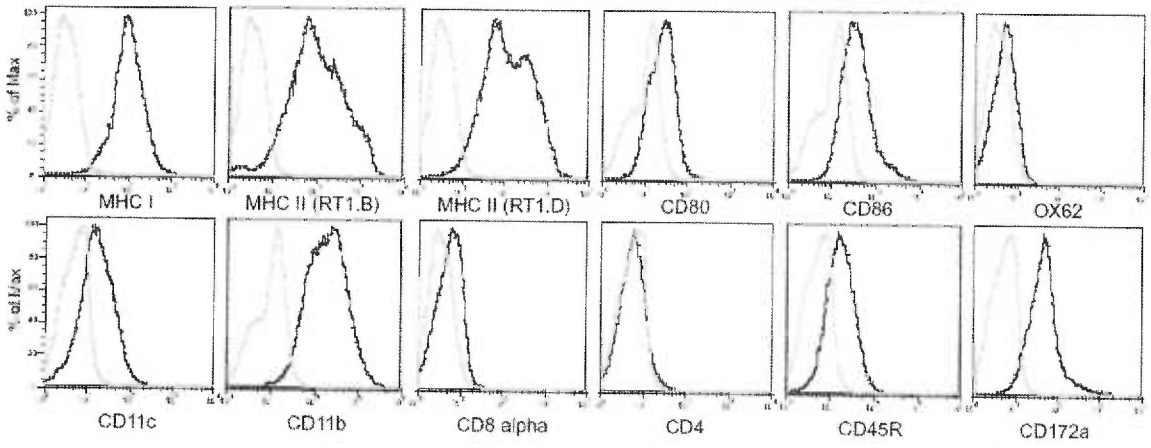


Figure 6. Rat bone marrow derived DC cell surface marker expression

DC cultures were generated from total bone marrow cells isolated from the femurs of adult male Lewis rats and cultured for 9 days in complete media plus GM-CSF and IL-4. Nonadherent cells were removed by washing, and the remaining adherent DCs were incubated for 24 hours in fresh media with or without LPS. Cells were analyzed at 24 hours post treatment by flow cytometry for cell surface expression of CD86, CD80, MHC I and II. Isotype control (grey), LPS treated cells (solid black), Mock treated-no LPS (dashed).

of GFP expression, however there were cells that displayed low but detectable levels of GFP. Approximately 61% of iDC infected at an MOI of 0.5 were GFP positive at 48hpi. Infection of mDC at an MOI of 0.5 yielded a lower infectivity rate compared to iDC (40% GFP+). The reduction in the total number of GFP positive cells coupled with the 44% reduction in MFI in infected mDC cultures compared to iDC cultured in parallel suggests a reduced susceptibility of mDC to RCMV infection relative to iDC. In the remainder of this study we further examined RCMV infection of iDC.

To determine whether RCMV productively infects BMDCs, we analyzed growth of the virus in these cells. For this experiment, DCs were infected with RCMV-WT or RCMV-GFP at an MOI equal to 1 and harvested at 1, 2, 3, 5, and 8 dpi. The samples were stored at -80°C until analysis. Titration of the combined cellular and supernatant fractions was performed by standard plaque assays. Viral replication was minimal in these cells, which mimics the growth of HCMV in DCs. The amount of infectious virus from the infected DCs was similar for both the RCMV-WT and RCMV-GFP viruses and the levels peaked at 3 dpi (Figure 7C).

RCMV reduces expression of DC surface markers critical to antigen presentation.

DC induced activation of T cells depends upon antigen presentation in the context of both MHC and costimulatory molecules. To investigate the effects of RCMV infection on antigen presentation and DC activation, iDCs were infected at an MOI of 1.0 or 0.1 with either RCMV-WT or RCMV-GFP. As shown in Figure 8

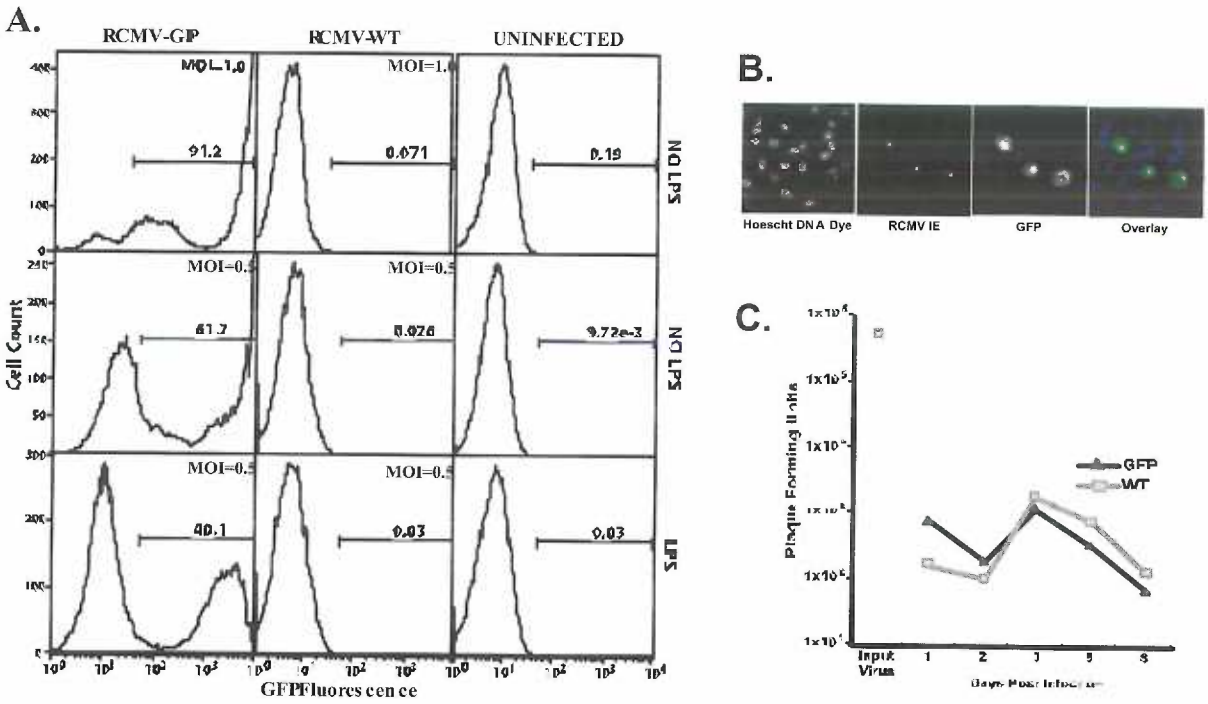


Figure 7. Rat BMDCs are highly susceptible to RCMV infection

A. BMDCs were infected 24h post LPS treatment (mDC) or mock (iDC) at an MOI=1.0 (top panel) or 0.5 (middle and bottom panels) for 48h and then assessed by flow cytometry for GFP expression. Gate numbers represent the percent of the population positive for GFP.

B. BMDC were infected with RCMV-GFP. At 48 hours post-infection, the cells were fixed with 1% paraformaldehyde, permeabilized and stained with anti-RCMV IE antibody (red) and with Hoescht (blue), GFP (green). Mag=60x.

C. Growth kinetics of RCMV-WT (squares) and RCMV-GFP (triangles) in BMDCs. At the indicated time points after infection (days post-infection) the presence of virus (combined intracellular and extracellular) in the cultures was determined by standard plaque assays on rat RFL6 fibroblasts. Viral titers are expressed as plaque forming units per ml and represent the average of three replicates.

cell surface expression of the costimulatory molecules CD80 and CD86 as well as MHC I and II was analyzed at 48hpi by flow cytometry. Infection of iDCs resulted in a 94% and 89% drop in both MHC II and I (respectively) MFIs relative to uninfected cells (Figure 8A&B). DC infected with RCMV-WT and -GFP displayed equivalent levels of MHC on the cell surface, suggesting that the deletion of ORFs r145-147 or the expression of eGFP does not affect RCMV-mediated down regulation of MHC. CD44H, hyaluronic acid receptor, expression was analyzed as a control for a possible viral induced global down regulation of host cell surface markers (Figure 8E). The levels of CD44H were unchanged in infected versus uninfected cells. This finding suggests that the RCMV-induced reduction in MHC expression is a specific event and not the result of a global down regulation of cell surface molecules.

Expression levels of the costimulatory molecules, CD80 and CD86, were also examined as possible RCMV targets of the host cell antigen presentation pathway. While uninfected iDCs maintained low level CD86 expression, cells infected at an MOI=1.0 with either RCMV-WT or -GFP up regulated surface expression of CD86 (Figure 8C). Cell surface expression of CD80 was examined in the context of RCMV-GFP infection (MOI=0.1); iDCs from the infected culture were gated on GFP expression. CD80 expression in infected (GFP positive) cells was reduced to isotype control levels, while uninfected (GFP negative) cells had slightly elevated levels of the costimulatory molecule compared to cells from uninfected cultures (Figure 8D). The dramatic reduction in cell surface

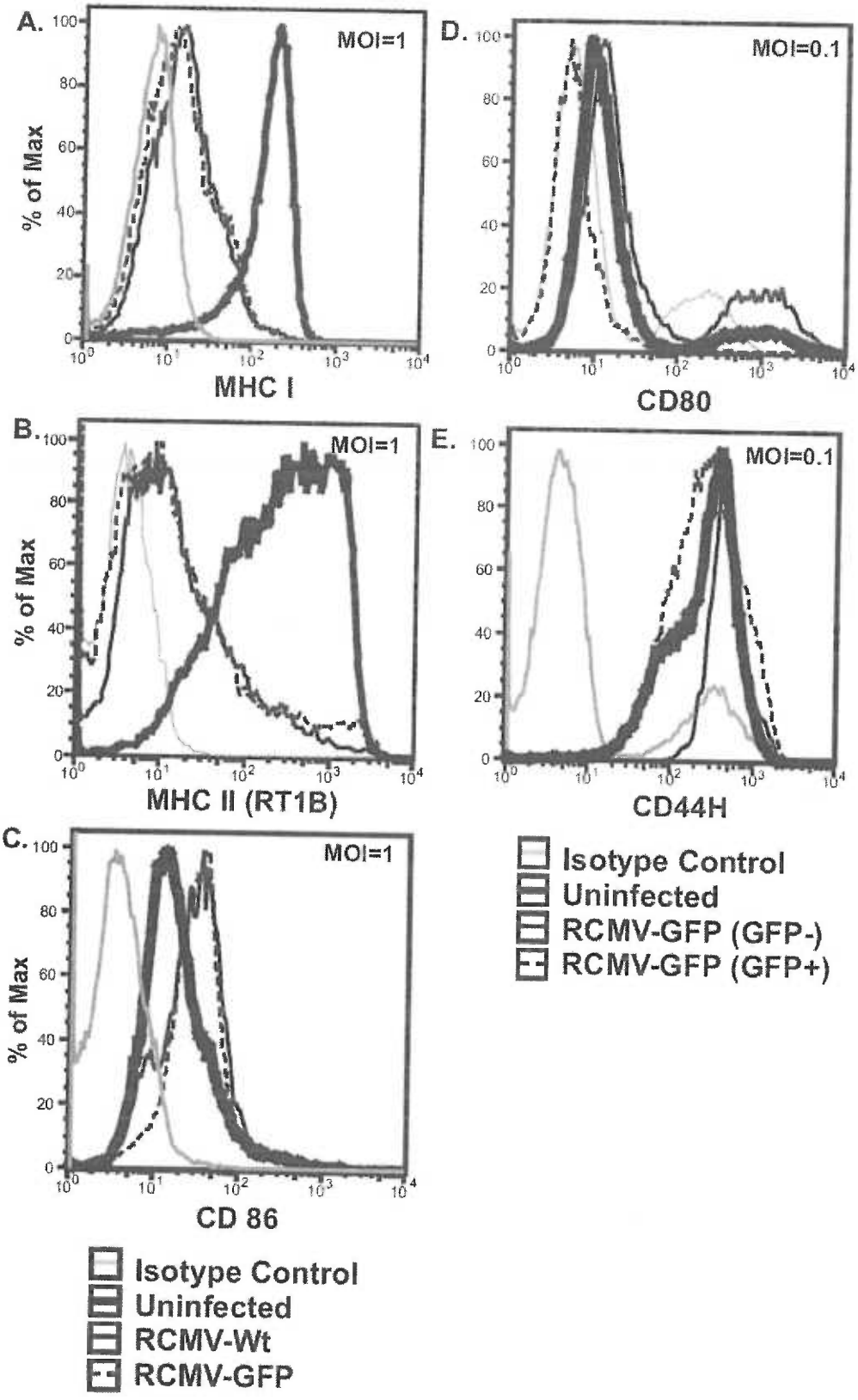

A.
MOI=1
% of Max
MHC I
D.
MOI=0.1
CD80
B.
MOI=1
MHC II (RT1B)
E.
MOI=0.1
CD44H
C.
MOI=1
CD 86
Isotype Control
Uninfected
RCMV-GFP (GFP-)
RCMV-GFP (GFP+)
Isotype Control
Uninfected
RCMV-Wt
RCMV-GFP

Figure 8. RCMV down regulates MHC I and II cell surface expression

A-C. iDCs were infected at an MOI equal to 1.0 with either RCMV Wt (thin black line), RCMV GFP (dashed black line) or mock (thick black line) for 48hrs and surface stained for MHC I (A), MHC II RT1B (B) or CD86 (C). Isotype controls were depicted with a thin grey line. In panels D and E, iDCs infected with RCMV-GFP at an MOI=0.1 were stained for surface expression of CD80 (D), MHC II RT1D (E) or CD44H (F). Infected cultures were gated for GFP expression [GFP positive cells (thin dashed line), GFP negative cells (thin black line), mock treated cells (thick black line), and isotype controls (thin grey line)].

expression of MHC I, II and the costimulatory molecule CD80 to isotype control levels in RCMV infected BMDC suggests a specific targeting of the host cell antigen presentation pathway. The increased expression of CD86 and static level of CD44H in infected BMDC further suggests that the loss of MHC and CD80 is not the result of a universal down regulation of cell surface marker expression, but rather a specific viral strategy aimed at subversion of the host cell adaptive immune response.

RCMV infection of iDC blocks CD4 T cell proliferation in an allogeneic mixed leukocyte reaction.

To determine the impact of RCMV-mediated down regulation of cell surface MHC II on DC function as antigen presenting cells, mixed leukocyte reactions were performed on infected versus uninfected iDCs. CD4 T cell proliferation (as monitored by CFSE dilution) was used as a measure of CD4 T cell activation. BMDC were generated, as described above, from Lewis strain rats. These cells were mock infected or infected with RCMV-WT for 72h at MOI=1.0, and then incubated with CFSE labeled splenocytes isolated from the allogeneic rat strain ACI. BMDC and splenocytes were cocultured for 5 days before analysis by flow cytometry. Nonadherent cells were taken from the culture and stained for CD4. T cell proliferation was measured as a loss of CFSE intensity. Data is represented as the percent of total CD4+ cells that proliferated. A greater than 20-fold reduction in T-cell proliferation was observed in the allogeneic T-cells added to the infected iDC cultures compared to the uninfected cells (Figure 9).

This finding suggests that iDCs infected with RCMV are functionally impaired and lack the ability to induce T-cell proliferation.

RCMV depletes both cell surface expression and intracellular MHC II in DCs.

To determine whether the RCMV-induced down regulation of MHC II surface levels was due retention of these molecules in intracellular compartments, we assessed the total levels of MHC II in infected iDCs by immunofluorescence staining and western blotting. BMDC infected with RCMV-GFP at an MOI=1.0 were fixed and permeabilized at 48 hpi. Cells were stained for MHC II and visualized by epifluorescence using a deconvolution microscope. The infected, GFP positive, iDC had undetectable levels of MHC II staining, while GFP negative cells in the same field of view showed both a diffuse cytoplasmic and concentrated plasma membrane staining for MHC II (Figure 10A). The GFP positive cells stained similarly for CD44H compared to uninfected cells. Immunofluorescence images shown in Figure 10B of iDC infected with RCMV-WT further confirm the RCMV-GFP IF staining. BMDC were infected at an MOI=0.5. Infected cells were stained for IE (red) and MHC II (green). The absence of MHC II in IE positive cells is in agreement with the reduction of detectable MHC II in RCMV-GFP positive cells. The mutually exclusive staining patterns of MHC II and RCMV IE/GFP is evocative of the flow cytometry data in which cell surface levels of MHC II were nearly undetectable in infected iDC. Similarly, DCs infected with RCMV were completely devoid of MHC II as

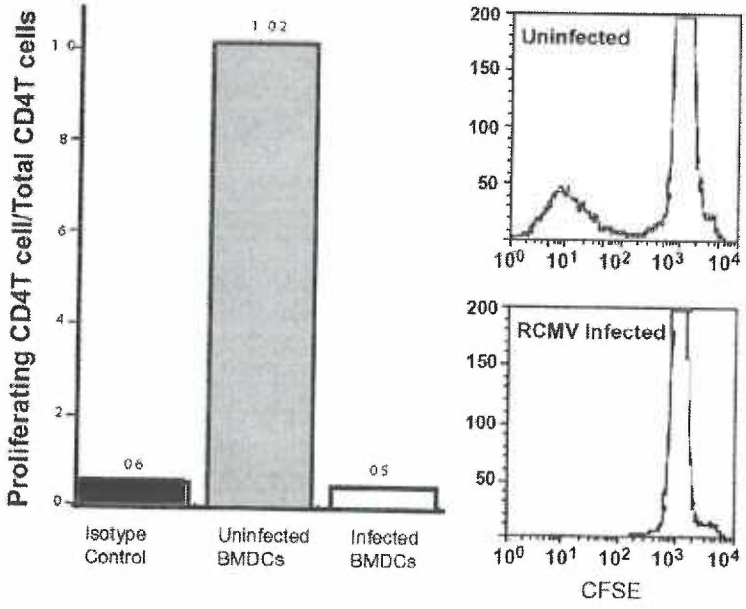


Figure 9. RCMV infected BMDC are unable to induce allogeneic CD4 T cell proliferation in a mixed leukocyte reaction

BMDC isolated from Lewis strain rats were infected with RCMV-Wt for 72h, and cocultured with CFSE labeled splenocytes taken from ACI strain rats for 5 days. Non-adherent cells were stained with anti-CD4 Ab and analyzed by FACS. CD4+ T cell proliferation was measured by CFSE dilution. T cell proliferation is represented as the percent of total CD4+ T cells that have divided. Isotype control (black fill), Uninfected (dark grey fill), RCMV infected (white fill). Shown in the right panels are histograms demonstrating CFSE fluorescence in CD4+ T cells mixed with infected or uninfected DCs.

determined by western blot analysis (Figure 11B).

RCMV-mediated MHC down regulation occurs at a post-transcriptional step

To determine the kinetics of the RCMV-mediated down regulation of MHC I and II, a time course analysis of MHC staining following virus infection was undertaken. For this experiment, in order to identify infected vs. uninfected cells within the same culture, RCMV-GFP was used to infect iDC at an MOI=0.1. Cells were analyzed for surface expression levels of MHCI and II at 12, 24, 48, and 72 hpi, and gated on GFP expression to examine direct effects of RCMV infection and GFP negative cells to study indirect effects. The virus-mediated MHC II down regulation becomes detectable between 12 and 24hpi (Figure 11A). However, the most striking drop in MHC II cell surface expression occurs at 24hpi (83% drop in MFI of infected versus uninfected cells), which corresponds to the timing of early viral gene expression. By 72hpi cell surface levels approach isotype control levels indicating that RCMV infection removes MHC II to background levels. The kinetics of MHC I down regulation is delayed in comparison to MHC II. Cell surface levels drop in the infected cells between 24 and 48hpi, further declining (greater than 40% reduction) by 72hpi (Figure 11A). Importantly, MHC I and II are only down regulated in the GFP positive (infected) cells suggesting that this effect is actively mediated by virus replication and not a soluble factor found within the inoculum or secreted as a result of infection. We

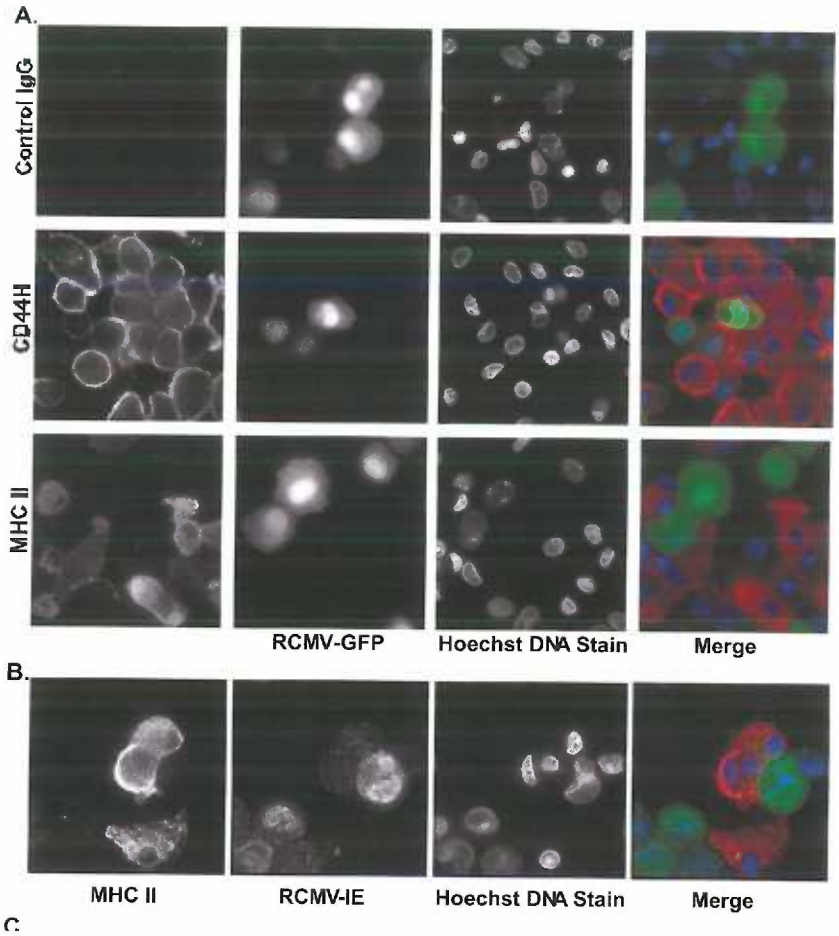


Figure 10. Intracellular MHC II is undetectable in RCMV infected BMDC

A. BMDCs were infected with RCMV-GFP. Cells were fixed, permeabilized and stained 48hpi with either an IgG isotype control antibody or antibodies directed against CD44H or MHC II (red). Deconvolution microscopy was used to visualize the stained cells. Mag=60x.

B. BMDCs were infected with RCMV-Wt. Cells were permeabilized and stained 48hpi with Hoechst (blue), MHC II (green) and anti-RCMV IE antibody (red). Deconvolution microscopy was used to visualize the stained cells. Mag=60x.

FIGURE 11.

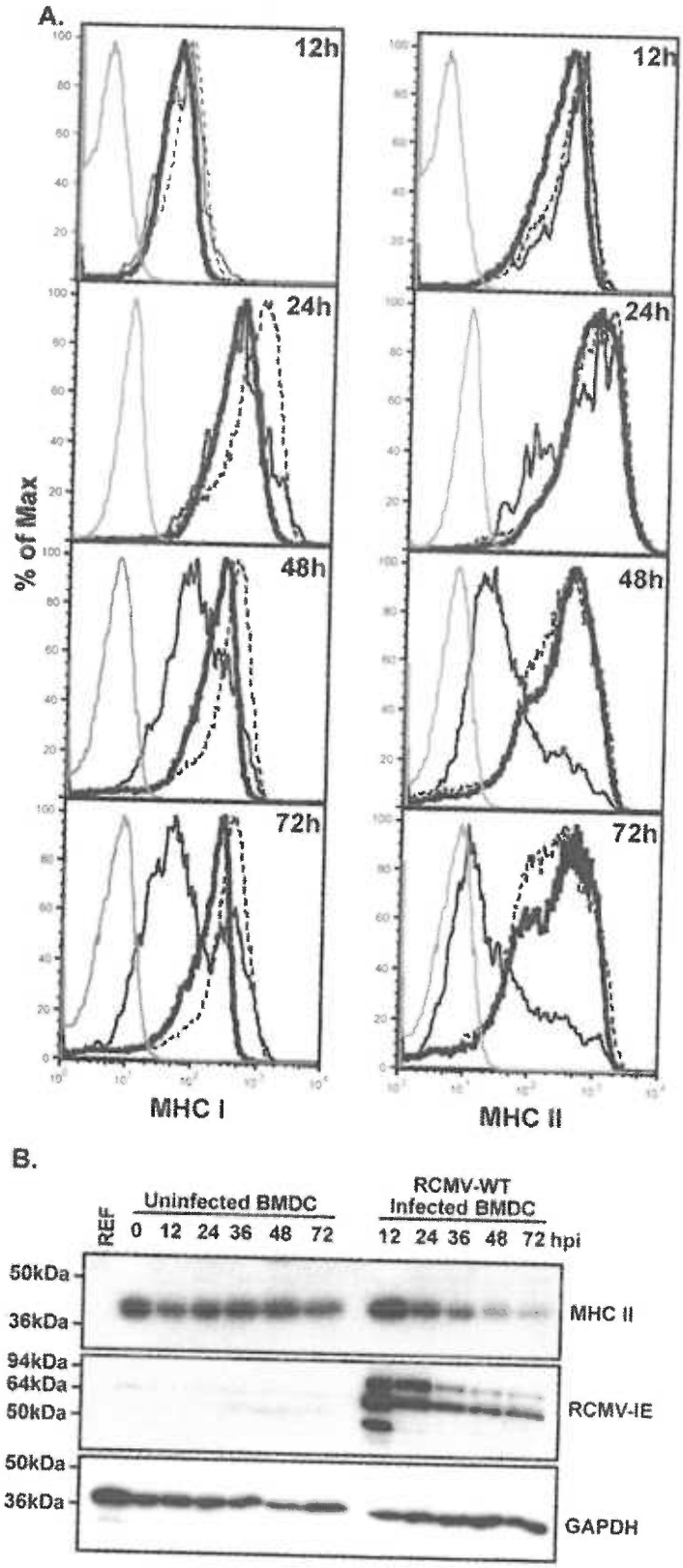

A.
12h
24h
48h
72h
% of Max
MHC I
MHC II
B.
REF
Uninfected BMDC
0 12 24 36 48 72
RCMV-WT
Infected BMDC
12 24 36 48 72 hpi
50kDa
36kDa
MHC II
94kDa
64kDa
50kDa
RCMV-IE
50kDa
36kDa
GAPDH

Figure 11. RCMV induces a stable depletion of cell surface MHC I and II

A. BMDC were infected with RCMV-GFP at an MOI=0.1 and harvested at the times indicated for analysis via FACS. Uninfected cells were harvested in parallel at the indicated times. Isotype control (grey line), Uninfected cells (thick black line), RCMV infected, GFP positive cells (thin black line), BMDC from the same infection culture but uninfected, GFP negative (dashed black line).

B. Western blot analysis of MHC II depletion in RCMV-infected BMDC. BMDC were infected with RCMV-WT at an MOI=1 and the cells were harvested in Laemmli's sample buffer at the indicated time points after infection. Uninfected rat RFL6 cells and uninfected BMDCs served as controls. Blots were stained for MHC II, RCMV-IE or GAPDH.

also analyzed total levels of MHC II by western blotting at 12, 24, 36, 48, and 72 hpi in order to determine whether the kinetics of the observed degradation of total MHC II corresponds to the decrease in surface expression. As shown in Figure 11B, initially at 12 hpi MHC II is slightly up regulated in the infected cultures. However, MHC II degradation begins between 24 and 36 hpi occuring at the time of the reduction in surface expression. By 48 hpi the levels have dropped by 90%. This data demonstrates that the reduction in MHC II surface expression corresponds to a similar reduction in total cellular MHC II levels.

To elucidate the mechanisms of RCMV-mediated down regulation of MHC, we determined the effects of RCMV infection on MHC transcription and translation. To determine whether RCMV infection affects transcription of MHC in BMDC, we used quantitative RT-PCR TaqMan to compare the mRNA levels of MHC I and II at 12, 24, and 36 hpi. The expression of both MHC I and II was slightly elevated at 12 hpi. However, by 24 hpi the levels of gene expression returned to those levels observed in uninfected cells (Figure 12A). This finding indicates that RMCV infection does not affect the transcription of MHC. Therefore, since a massive depletion of MHC II occurs in the infected cells beginning at 24-36 hpi, we next explored whether RCMV modulated translation of MHC II. For this assay, BMDC were infected with RCMV for 24 hours and then metabolically labeled in a pulse-chase experiment and the cellular lysates were immunoprecipitated with antibodies directed against MHC II (RT1B) and

FIGURE 12.

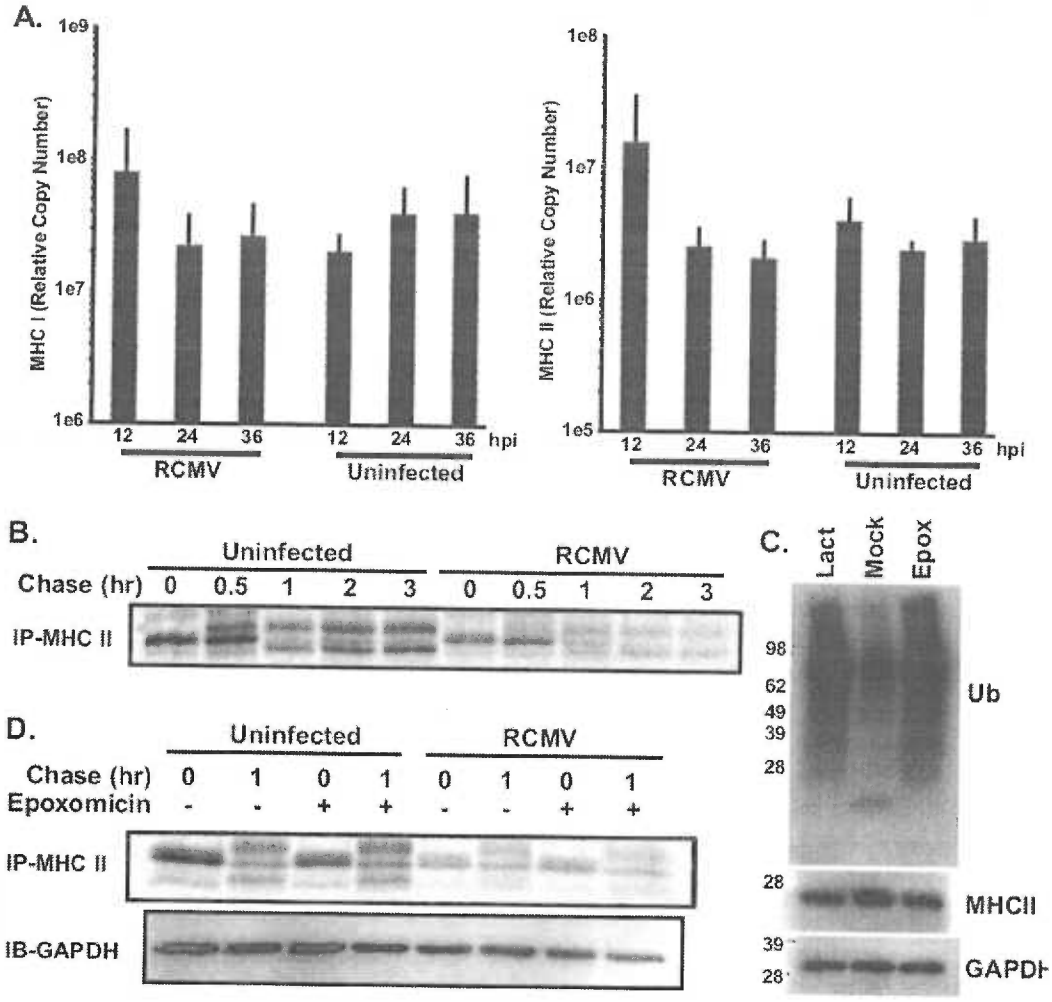

A.
MHC I (Relative Copy Number)
1e9
1e8
1e7
1e6
12 24 36 12 24 36 hpi
RCMV
Uninfected
MHC II (Relative Copy Number)
1e8
1e7
1e6
1e5
12 24 36 12 24 36 hpi
RCMV
Uninfected
B.
Uninfected
RCMV
Chase (hr) 0 0.5 1 2 3 0 0.5 1 2 3
IP-MHC II
C.
Lact
Mock
Epox
98
62
49
39
28
Ub
28
MHCII
39
28
GAPDH
D.
Uninfected
RCMV
Chase (hr) 0 1 0 1 0 1 0 1
Epoxomicin - - + + - - + +
IP-MHC II
IB-GAPDH

Figure 12. RCMV Infection does not alter gene expression of MHC I and II in rat BMDCs

A. RT-PCR TaqMan was used to detect expression of MHC I and MHC II in uninfected and RCMV-infected rat BMDC at 12, 24, and 36 hpi (n=3). RT-PCR TaqMan detection mRNA from the gene for the ribosomal protein L32 was used to normalize the expression data (n=4).

B. Pulse chase analysis was performed in mock- and RCMV-infected BMDC at 24hpi. Cells were starved and then labeled with ^{35}S-methionine/cysteine at 0.2mCi/ml for 10 min. After the pulse, cells were diluted in chase media containing an excess of cold cysteine and methionine and then washed. Cells were then chased for 0.5, 1, 2 or 3 hrs at 37°C and then harvested by lysing the cell pellets in lysis buffer. MHC II was immunoprecipitated using the indicated antibody for 1hr at 4°C, followed by a 2hr incubation with Protein A/G beads. Beads were washed and resuspended in sample buffer and analyzed by SDS-PAGE and visualized by autoradiography.

C. Western blot analysis of Ub in BMDC treated with epoxomicin (100nM) or lactacystin (50μM) for 36hrs. Cells were harvested in sample buffer at 36hrs post treatment. Untreated BMDCs (mock) served as controls. Blots were stained for Ubiquitinated proteins, MHC II RT1B and GAPDH.

D. Pulse chase analysis was performed in mock- and RCMV-infected BMDC cultures treated with or without the proteosomal inhibitor epoxomicin (100nM). Cells were starved and then labeled with ^{35}S-methionine/cysteine at 0.2mCi/ml for 10min. After the pulse, cells were diluted in chase media containing an excess of cold cysteine and methionine and then washed. Cells were then chased for 1hr at 37°C and then harvested by lysing the cell pellets in lysis buffer. MHC II was immunoprecipitated using the indicated antibody for 1hr at 4°C, followed by a 2hr incubation with Protein A/G beads. Beads were washed and resuspended in sample buffer and analyzed by SDS-PAGE and visualized by autoradiography. A fraction of the cellular lysate for each sample was also probed by western blotting for GAPDH to ensure equal loading.

analyzed by SDS-PAGE. The immature (EndoH sensitive) form of MHC II was present in the pulse samples for both the uninfected and infected cell lysates (Figure 12B). However, there was a reduction in the accumulation of labeled MHC II in the RCMV infected BMDCs. This was accompanied by a similar reduced level of MHC II in the chase of the infected cells. In order to establish whether this down regulation was mediated by the proteosome, epoxomicin was used to treat the cells prior to and during the pulse-chase. Western blots to measure the increase in poly-ubiquinated species upon proteasome inhibition was used to confirm the effective concentration of lactacystin and epoxomicin in the rat BMDCs (Figure 12C). Epoxomicin treatment did not affect MHC II protein levels observed in both the pulse and chase samples from the uninfected or infected cells (Figure 12D). This suggests that while there is a post-transcriptional reduction in MHC II it is not mediated by proteasomal degradation.

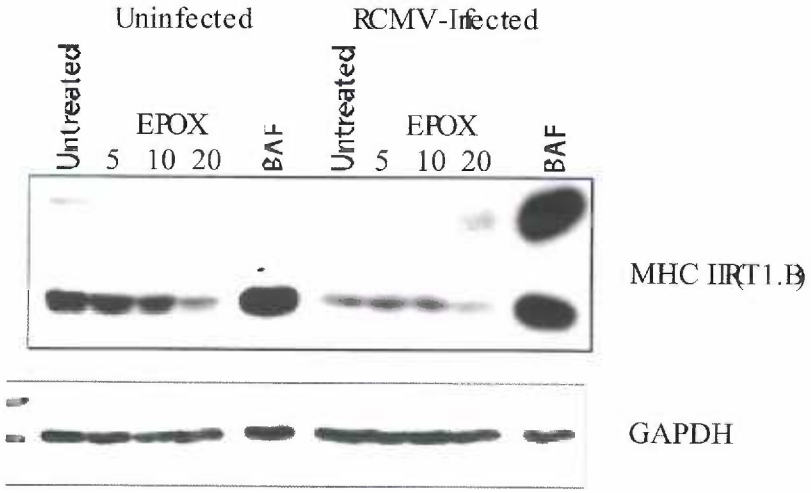
RCMV degradation of MHC II is inhibited by increasing endosomellysosomal pH.

While the post-transcriptional effect on MHC II synthesis in RCMV infected cells at 36 hpi suggests a mechanism for the sustained reduction, this mechanism would not be sufficient for the rapid degradation of MHC II occurring by 24 hpi. We reasoned that one possible mechanism for this massive depletion of cell surface MHC II is through increased recycling and degradation in the infected cells. To test this hypothesis, we performed western blot analysis for MHC II in cellular lysates from infected and uninfected BMDCs treated at 12hpi with

bafilomycin for an additional 36hpi. Bafilomycin inhibits the proton pump associated with endosomes and lysosomes ultimately inhibiting the pH sensitive proteinases found in these vesicles. Bafilomycin inhibited the degradation of MHC II observed in the infected cells (Figure 13A). Bafilomycin also increased accumulation of MHC II in uninfected cells. Interestingly, a SDS-stable form of MHC II was only detected in the bafilomycin treated RCMV infected BMDCs. Epoxomicin treatment failed to recover MHC II in the RCMV-infected cells, confirming our findings above (Figure 12D) that indicate that RCMV-degradation of MHC II is not due to proteasomal degradation. To confirm that the effect of bafilomycin on preventing RCMV-mediated MHC II degradation and to visualize the cellular location of MHC II accumulation, we visualized immunofluorescence stained uninfected and RCMV-infected BMDCs with and without bafilomycin treatment by deconvolution microscopy. For this assay, BMDCs were infected with an MOI=0.5 to ensure that at least 50% of the cells were infected. As demonstrated above, MHC II was present in the uninfected cells but not in the GFP+ infected BMDCs in the same culture (Figure 13-lower panel). However, MHC II was observed in the infected cells when treated for 24 hrs with bafilomycin. In these cells MHC II was predominantly in large vesicles suggesting that RCMV promotes the recycling of MHC II, which is then degraded in an endocytic or lysosomal compartment.

Figure 13.

A.


Uninfected
RCMV-Infected
Untreated
EPOX
5
10
20
BAF
Untreated
EPOX
5
10
20
BAF
MHC II(RT1.B)
GAPDH

B.

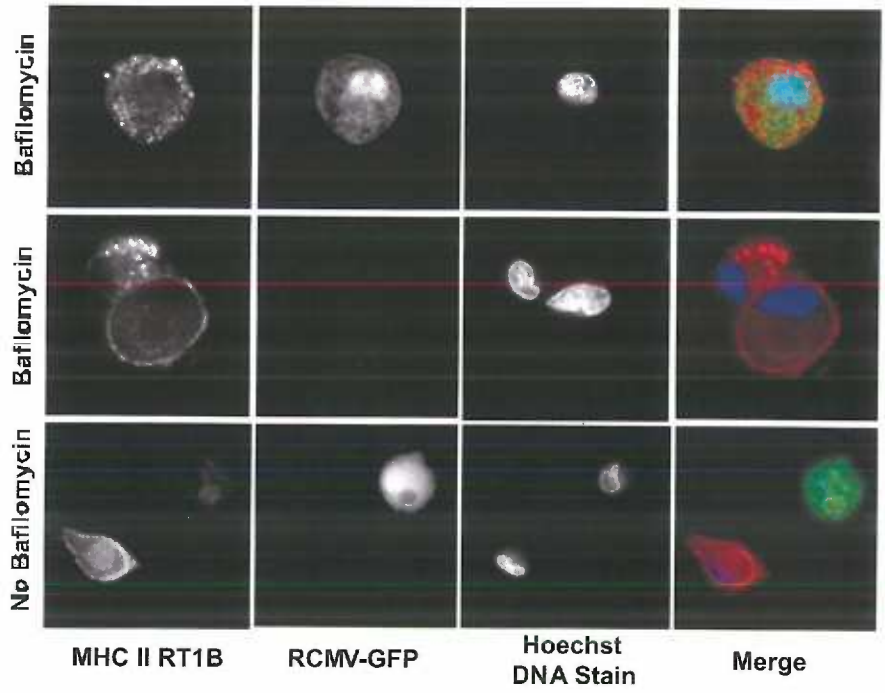

Bafilomycin
Bafilomycin
No Bafilomycin
MHC II RT1B
RCMV-GFP
Hoechst
DNA Stain
Merge

Figure 13. RCMV down regulation of MHC II is sensitive to treatment with bafilomycin.

A. Western blot analysis of MHC II depletion in RCMV-infected BMDC treated with epoximicin (5, 10, and 20 nM) or bafilomicin (100nM). BMDC were infected with RCMV-WT at an MOI=1 and the cells were harvested in Laemmli's sample buffer at the indicated time points after infection. Uninfected rat RFL6 cells and uninfected BMDCs served as controls. Blots were stained for MHC II RT1B or GAPDH.

B. BMDCs were infected with RCMV-GFP for 12hrs and then treated with bafilomicin (100nM) for an additional 36 hrs. Cells were fixed, permeabilized, and then stained with antibodies directed against MHC II RT1B (red) and Hoescht DNA dye (blue). Infection was detected by GFP expression (green). Deconvolution microscopy was used to visualize the stained cells. Mag=60x.

RCMV down regulation of MHC I & II requires IE/E gene expression.

RCMV mediated MHC down-modulation could be mediated by a number of factors including a cellular response to RCMV binding and entry, a component of the virus inoculum or of the virion itself, or a protein encoded in the viral genome requiring viral replication for expression. To determine whether virus replication was necessary for MHC down regulation, BMDC were infected at an MOI=1.0 with either RCMV-WT or UV inactivated RCMV-WT from the same viral preparation. Infected and mock treated cells were examined 48hpi by flow cytometry for the presence of MHC I and II (Figure 14A). MHC I cell surface expression was slightly increased (MFI equal to 103 versus uninfected MFI equal to 71.1) in cells infected with UV inactivated virus, while MHC II levels were relatively unchanged (MFI=70.1) compared to uninfected cells (MFI=79.8). These data demonstrate that neither a component of the viral inoculum, a protein component of the virion itself, nor viral binding/entry mediate the effect observed on MHC down regulation induced by RCMV infection.

Next, we determined whether late viral gene expression was required to induce MHC down regulation. RCMV infected iDCs were treated with Foscarnet, an inhibitor of RCMV DNA synthesis, which blocks virus late gene expression and viral replication. MHC I and II cell surface levels were reduced regardless of Foscarnet treatment in the infected cells and were unaffected in mock-infected cells (Figure 14B). Although basal levels of MHC II (on uninfected iDC) were reduced in cells treated with Foscarnet, versus uninfected untreated iDC,

infection in the presence or absence of the inhibitor continued to reduce the level of cell surface MHC II (by greater than 65%). The continued loss of MHC expression in the presence of Foscarnet suggests that RCMV-induced MHC depletion is not mediated by late viral gene transcription. Together, these results support the hypothesis that the RCMV-induced depletion of MHC I and II is mediated by a viral immediate early or early gene product.

Soluble factors do not mediate RCMV induced MHC down regulation.

Previous studies investigating HCMV and MCMV induced MHC II manipulation have implicated both a viral homologue of IL10 (UL111a) and the induction of cellular IL10, respectively (38, 213, 256). Since the RCMV genome lacks an UL111a homologue, a possible role for cellular IL10 or other secreted factors in the modulation of MHC II surface expression was explored. For this experiment, supernatants collected from RCMV-WT infected iDC were clarified by ultracentrifugation and these cell- and virus-free supernatants were incubated with uninfected iDC for 48hrs. Surface levels of MHC II were slightly elevated in cells treated with supernatants from infected cells compared to those cells treated with supernatants from uninfected DC (Figure 14C). One could argue that a soluble factor released by an infected cell may have a limited stability in culture. If this were true, one would predict that the uninfected cells within the same infected DC culture would also down regulate MHC II from the cell surface. However, that is not the case since uninfected cells from low MOI infected cell cultures, wherein not all of the cells are infected, do not down regulate MHC

molecules from their surface (shown in Figures 8E&11A). As an added control for whether virus-derived or -induced cellular secreted IL10 mediated the effect on MHC down regulation, infected and uninfected cells were incubated with saturating concentrations of a neutralizing antibody to rat IL10 (data not shown). Both infected and uninfected cells treated with anti-IL10 neutralizing antibody failed to elicit any significant changes in their MHC levels compared to antibody-untreated cultures. These findings in combination with our low MOI experiments presented in Figures 8E&11A argue against a soluble factor causing the depletion of MHC molecules from RCMV-infected DC.

DISCUSSION

In the current report an RCMV/DC infection system was utilized to explore the effects of CMV infection on DC function. We demonstrate for the first time that rat BMDC are highly susceptible to RCMV and that infection significantly down regulates surface levels of CD80, MHC I and MHC II, while other cell surface markers such as CD44H are not down regulated suggesting that the effect is specific for a subset of cell surface markers. A greater than 90% reduction of MHC II from both the cell surface and intracellular pools in RCMV infected rat DCs was observed. The significance of the loss of MHC and the costimulatory molecule, CD80, was underscored when a functional impairment in DC mediated antigen presentation was confirmed in a mixed leukocyte reaction. RCMV does not affect MHC transcription but may have a small effect on translation at a post-transcriptional step. The RCMV-mediated degradation of MHC II was inhibited by

Figure 14.

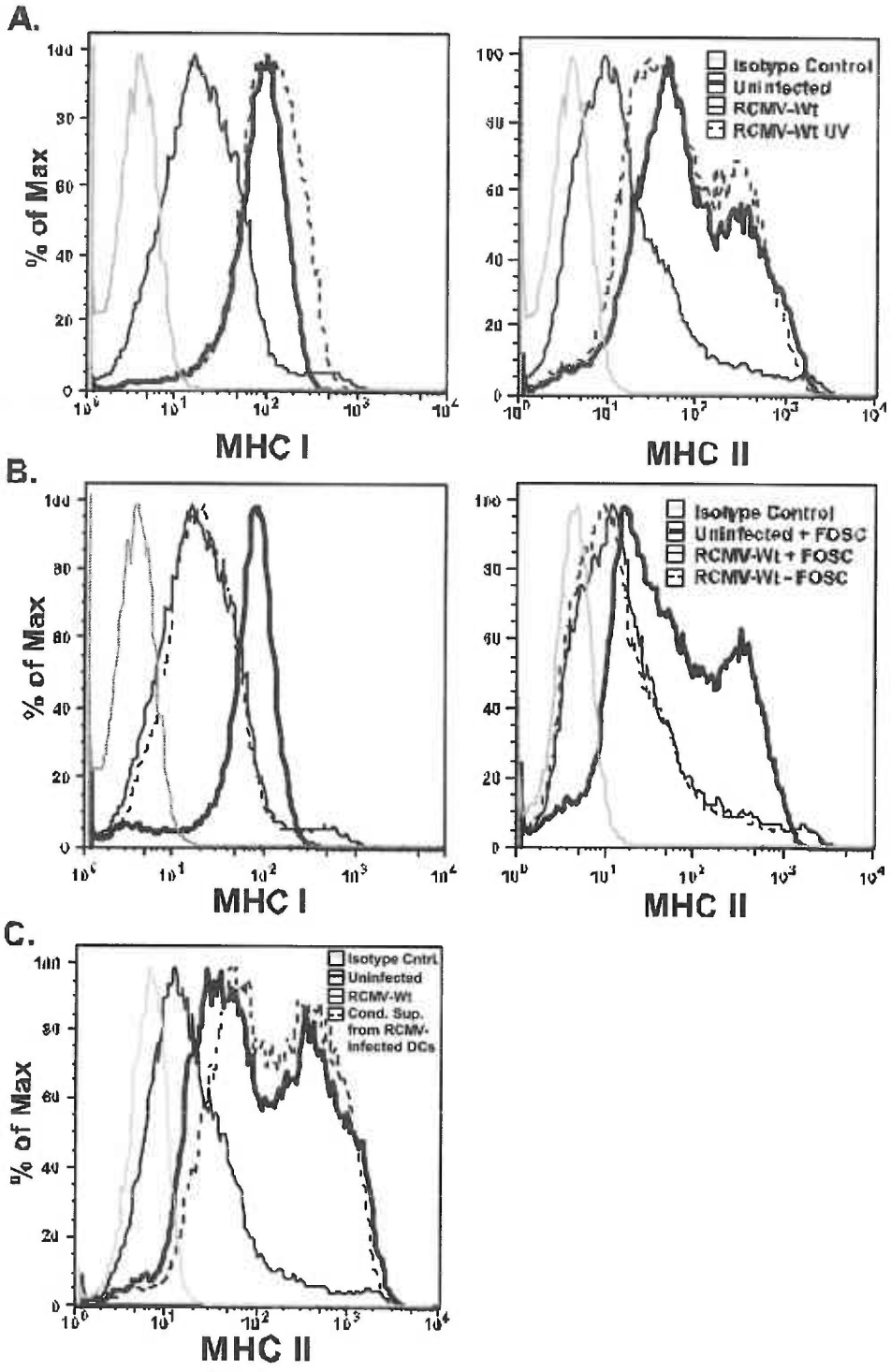

A.
% of Max
MHC I
MHC II
Isotype Control
Uninfected
RCMV-Wt
RCMV-Wt UV
B.
% of Max
MHC I
MHC II
Isotype Control
Uninfected + FOSC
RCMV-Wt + FOSC
RCMV-Wt - FOSC
C.
% of Max
MHC II
Isotype Cntrl
Uninfected
RCMV-Wt
Cond. Sup. from RCMV-infected DCs

Figure 14. RCMV induced reduction in MHC expression is not mediated by viral binding, entry, late gene expression or by a secreted factor

A. BMDC were infected at an MOI=1 with RCMV-Wt (thin black line) or Wt-UV inactivated (dashed black line) or mock infected (thick black line) for 48h and analyzed by FACS for cell surface expression of MHC I and II. Isotype control (grey).

B. BMDC were pretreated with Foscavir or mock, then infected at an MOI equal to one with RCMV-Wt. Isotype control (grey), Uninfected + Foscavir (thick black line), Infected + Foscavir (thin black line), Infected – Foscavir (dashed black line).

C. BMDC were infected with RCMV-Wt (thin black line) or mock (thick black line), or conditioned supernatants from RCMV infected BMDCs (dashed black line), for 48hrs and then assessed for cell surface expression of MHC II. Isotype control (grey line), RCMV-Wt infected (thin black line), uninfected (thick black line), conditioned supernatant treatment (dashed black line).

the ATPase/proton pump inhibitor bafilomycin, suggesting that the virus promotes destruction of MHC II in endosomes/lysosomes by cellular or viral pH-dependent. Unlike MCMV, the RCMV-mediated reduction of MHC was not caused by secreted factors since supernatants from infected cells failed to induce a reduction in MHC surface expression. Furthermore, when cells infected at low MOI (equal to 0.1) with RCMV-GFP were gated for GFP expression, only the GFP positive cells were depleted of MHC II. In the same infected DC culture the uninfected GFP negative cells had high levels of MHC II. Viral replication was required for the down regulation of MHC I and II as DCs infected with UV-inactivated RCMV displayed MHC levels that paralleled uninfected cells. The effect on MHC I and II surface expression was mediated by an immediate early or early gene, other than R145-147, since both the treatment with Foscarnet (inhibits late viral gene expression) and infection with the RCMV-GFPΔR145-147) virus failed to block the down regulation. The high susceptibility of the DC to infection coupled with the stable and near complete loss of MHC all highlight the RCMV-BMDC infection model as an extremely useful tool to study the role of CMV infection in the down regulation of MHC II.

MHC I down regulation is likely the most widely used means of viral immune evasion occurring in both RNA and DNA viruses. Down regulation of MHC I in CMV infected cells eliminates CTL recognition of infected fibroblasts *in vitro* (198). Although a dramatic enhancement in viral fitness *in vivo* remains to be linked directly to MHC I depletion, blockade of CD8+ T cell surveillance is likely to

assist in viral persistence or reactivation from latency in a particular cell type, as seen during HSV reactivation from latency (145). In this report we demonstrate that RCMV dramatically reduces MHC I surface expression to near background levels. A previous study by Hassink *et. al.* demonstrated that RCMV down regulates MHC I expression on the surface of RCMV infected fibroblasts (97). However, the effects of RCMV infection on MHC I expression as reported by Hassink *et. al.* were modest and transient, occurring only at 12 hpi. Here we demonstrate that RCMV infection causes a massive down regulation of MHC I surface expression in infected dendritic cells. While this is not the first report to demonstrate that RCMV infection affects MHC I surface levels, the data outlined in this study are consistent with the effects observed in HCMV, RhCMV, and MCMV infected cells. It is unclear why such a dramatic surface down regulation of MHC I occurs in RCMV-infected DCs but not in fibroblasts, however the distinct effects may reflect the differences in the cell types used for these assays.

While viral interference with the MHC I antigen presentation pathway has long been recognized as an adaptive strategy to evade CD8+ T cell recognition, the advantage of viral subversion of the MHC II pathway has only recently been the subject of investigation. MHC II is typically associated with the exclusive presentation of extracellular antigen, and presentation of viral antigen is more commonly associated with intracellular MHC I presentation, however, manipulation of MHC II could provide at least two advantages to a long-term persistent virus such as CMV. First, blockade of MHC II would inhibit APC

triggering of the adaptive arm of the immune system in response to phagocytosed infected cells (extracellular antigen). Second, it has been demonstrated that intracellular CMV antigen may be presented in the context of MHC II during an active infection of class II expressing cells (98). The build up of viral glycoproteins within the trans golgi network or acidic compartments in preparation for viral assembly may lead to a leakage of endogenous viral antigen into the MHC II compartment. CMV induced MHC II blockade is likely to be advantageous to establishing a persistent infection in both its ability to evade detection during a primary infection of an APC and the ability to suppress the CD4+ T cell responses including the downstream production of antibodies.

One of the most striking features of the RCMV-BMDC model system is the degree of MHC II depletion in infected dendritic cells. At 72 hpi, RCMV infected BMDC have a greater than 84% reduction in MHC II cell surface MFI compared to uninfected BMDC. Furthermore, intracellular MHC II was below the limit of detection by immunostaining in cells infected for 48h, indicating that in addition to the lack of MHC II on the cell surface it is either rapidly destroyed in the infected DC and/or RCMV blocks the synthesis of MHC II, however, the mechanisms remain to be elucidated. Reductions in MHC II cell surface expression ranging from 15-50% have been reported for HCMV-infected human monocyte derived DC and MCMV-infected mouse DC (9, 156, 177, 205, 290). Whether the variation in the degree of MHC depletion is due to specific differences in the origin of the cells, the differentiation state of the cells, the distinction between

HCMV and MCMV lab-adapted strains compared to a more wild type strain of RCMV, or simply due to an RCMV-specific phenomena, remains to be elucidated. However, CMV may have a more dramatic effect on MHC II down regulation in DC derived from bone marrow versus DC derived from peripheral blood monocytes, which underscores the importance of these cells in CMV pathogenesis. In any case, the RCMV model system provides a unique opportunity to explore the mechanisms and impact of CMV induced MHC II manipulation in the context of infection, which has previously proven problematic using the HCMV *in vitro* model systems. In fact, most of our knowledge about HCMV-mediated MHC II manipulation comes from studies that employed U373 cells transfected with the MHC constitutive transactivator, MIITA, to produce high levels of MHC II. Similarly, the study identifying UL83 as a modulator of MHC II utilized a system in which MHC II negative fibroblasts were induced to express the molecule upon stimulation with IFNγ (184). In these studies, isolated expression of UL83, US2 and/or US3 mediated the down regulation of MHC II in these induced expression systems (100, 184, 281). Similarly, HCMV infection of these cells also caused a significant decrease in MHC II expression. However, the degradation of MHC II was only decreased by about one-third when the MIITA transfected U373 cells were infected with a recombinant strain of HCMV (RV7186) lacking the IRS1-US11 region of the genome, suggesting that other regions of the genome were also involved in down regulating MHC II (36). These findings highlight the importance of developing direct infection-based systems for

the identification of the full complement of CMV genes involved in the modulation of MHC II.

A specific MCMV ORF that mediates MHC II down modulation has yet to be identified, however, both MCMV and HCMV have been shown to down regulate MHC II surface expression by inducing cellular IL10 (182, 213). HCMV also encodes a viral homologue of cellular IL10, cmvIL10 (UL111A), which is a structural and functional homologue of the cellular version (38, 132, 147, 206). Due to the inefficient infection rate of monocyte derived DC with HCMV, studies examining the effects of both cellular and viral IL10 have been performed with recombinant proteins and by transferring infected fibroblast conditioned supernatants onto DCs (38, 206). The effects of neither cellular nor cmvIL10 on MHC II expression in DCs has been explored in the context of an actual HCMV infection. In contrast to the ability of conditioned supernatants from HCMV and MCMV infected cells to transfer the effect of MHC II down regulation, we demonstrate in this report that RCMV conditioned supernatants alone do not deplete MHC II in uninfected DCs. The failure of RCMV cultured supernatants to modify MHC II was further substantiated when using the RCMV-GFP virus. In these experiments, GFP positive cells were low in MHC II expression, whereas GFP negative cells from the same infection culture expressed high levels of MHC II. Taken together, these results suggest the absence of a paracrine effect in RCMV induced MHC II down regulation. Studies investigating MHC II surface expression in macrophages shortly following HCMV infection found that infection

with UV-inactivated virus depleted MHC II surface expression (184, 185). Further examination of this effect in IFN-γ stimulated fibroblasts identified UL83 (pp65), the ORF encoding a prominent virion component, as a mediator of MHC II surface depletion in fibroblasts (184). While RCMV encodes a UL83 homologue, we show that the effect of MHC II down regulation in rat BMDC did not occur in cells infected with UV-inactivated virus. In addition, RCMV is not known to encode sequence homologues to the other two ORFs identified as MHC II modulators in HCMV, US2 and US3, suggesting an additional mechanism by which RCMV infection of DCs blocks MHC II surface expression and facilitates the degradation of intracellular MHC II (100, 281).

While the MHC II presentation pathway is likely a prime target of CMV manipulation due to its importance in coordinating the adaptive antiviral response, MHC II down regulation may have additional consequences in CMV induced disease. For example, the fact that CMV remains latent in myeloid lineage cells destined to become professional antigen presenting cells suggests that the effect on MHC II could have an important role in reactivation from latency. In addition, in vascularized solid organ and bone marrow transplantation HCMV seropositivity has long been identified as a major contributing factor in graft rejection (158). Our previous work showing that recipient immune response to allogeneic donor tissue is required for RCMV acceleration of transplant vascular sclerosis, the primary indicator of vascularized graft rejection, further highlights the interplay between the host immune system and CMV infection during transplant rejection

(189). Of particular interest is a recent study demonstrating that a subset of dendritic cells presenting alloantigen in the context of MHC II mediates allograft tolerance (183). The presentation of alloantigen by a subset of DC facilitated the generation of alloantigen specific regulatory T cells, which mediate graft tolerance (183). Human DC derived from patients undergoing active CMV infections are functionally impaired at inducing T-cell responses and express higher levels of proinflammatory cytokines (73, 289). Our finding that RCMV infection of BMDC nearly eliminates MHC II from the cell surface, taken together with the work of other groups suggests a possible mechanism by which RCMV accelerates allograft rejection by elimination of MHC II from the surface of infected DC diminishing their capacity to stimulate the development of regulatory T cells critical to maintaining tolerance to the donor tissue (73, 183, 289). The alteration of suppressor T cell development could tip the immune system from a state of tolerance to a state that enhances allograft rejection.

In summary, bone marrow derived DCs are highly susceptible to RCMV infection, and the process of active viral infection modulates the ability of these infected cells to mediate an effective immune response. As demonstrated in this report, the RCMV-BMDC infection model provides three advantages to the study of CMV infection of DC and the effect on CMV disease. First, the infection model will facilitate the identification of novel RCMV ORFs mediating CMV induced MHC II manipulation. The finding of previously unrecognized RCMV gene products affecting MHC II may lead to the discovery of both functional and sequence

homologues in HCMV and MCMV. Second, the high rate of infection of BMDCs with RCMV will facilitate mechanistic studies of CMV induced depletion of MHC II in infected DC. Third, the RCMV-BMDC model system will permit the investigation of CMV infection of DC and their possible role in graft rejection. A thorough analysis of the RCMV genes and mechanisms of MHC II down regulation will be imperative to dissect the role of Class II depletion in HCMV infected individuals.

Acknowledgements

We would like to thank Drs. Nagendra Hedge and Klaus Frueh for critically reviewing this manuscript and Drs. Bill Britt and David Johnson for thoughtful discussions.

2. FUTURE DIRECTIONS

MECHANISM OF LYSOSOMAL MEDIATED DEPLETION OF MHC II IN BONE MARROW DERIVED DENDRITIC CELLS

Three major pathways have been reported to describe maturation and peptide loading of MHC II (288). The classical pathway begins in the ER, where MHC II α/β heterodimers assemble onto preformed Ii chain trimers in a stepwise fashion, until a nonameric complex is formed. Signal sequences present in the tail of the Ii chain trimer direct transit out of the ER through the Golgi to the MHC II loading compartment (MIIC) where fusion with the late endosome provides a peptide pool suitable for MHC II loading. Acid activated proteases (*i.e.* Cathepsin S) present in the MIIC degrade Ii chain, leaving behind a small peptide fragment, termed CLIP, in the peptide binding groove. Association of the MHC II α/β heterodimer with HLA-DM stabilizes the heterodimer and catalyses the exchange of CLIP for antigenic peptide. Once loaded with peptide, the mature MHC II α/β molecule is stable and transported to the plasma membrane, a process that has been reported to reach completion between one and three hours post synthesis of MHC IIα. HCMV encoded US2 and US3 have been shown to interfere with both early and late steps of the classical pathway, inhibiting exit from the ER (and subsequently inducing the degradation of HLA-DRα) and stabilization of the α/β heterodimer through depletion of HLA-DM. The pulse chase experiments in our study do not support an RCMV induced disruption of the classical MHC II pathway, as an RCMV induced depletion of nascent alpha chain is not observed within the three hour chase period.

The second pathway of MHC II maturation directs nascent nonameric complexes via the Golgi to the plasma membrane, bypassing the MIIC altogether. These complexes can then be internalized in a clathrin and dynamin dependent manner and targeted to endosomes for further processing and removal of Ii (294). The third pathway involves recycling of mature MHC II peptide complexes from the plasma membrane in endocytosed vesicles (in a clathrin and dynamin independent manner) directed to the late endosomal compartment where either peptide is exchanged and recycled to the plasma membrane or the complex transits further to the lysosome for degradation (294). A recent flurry of studies has further delineated the steps involved in this third pathway. The Mellman lab discovered that expression of mature MHC II α/β heterodimers at the cell surface is regulated by ubiquitination of the MHC II beta chain in immature dendritic cells (237). Upon receipt of maturation stimuli, MHC II expression levels at the surface stabilize, and ubiquitination of the beta chain is no longer observed. Matsuki and colleagues built upon this observation and noted that in B cells MARCH-I, an E3 ubiquitin ligase, induced the ubiquitination of the beta chain (157). Furthermore, the half-life of cell surface MHC II was found to be dramatically increased in MARCH-I deficient B cells. More recently, IL10 was found to induce the expression of MARCH-I, which fits with the previous findings of IL10 induced depletion of MHC II in macrophages and dendritic cells treated with CMV cultured supernatants (50, 278). Finally, the down regulation of MARCH-I was found to be part of the maturation of human DCs leading to the stabilization of MHC II at the plasma membrane (50).

Several lines of evidence point to interference with the recycling pathway of MHC II as the most likely target for RCMV. The timing of induced MHC II depletion, at more than three hours post synthesis, after transit out of the ER, all suggest that the effect is mediated on mature MHC II, either at the plasma membrane, in recycling vesicles or in the MIIC shortly after peptide loading and before transit to the plasma membrane. The more dramatic effect observed on immature DCs as compared to LPS-matured DCs, suggests a role for RCMV in disrupting MHC II return to the plasma membrane during recycling. However, the fact that RCMV is still able to deplete MHC II from the plasma membrane of mature DCs argues in favor of additional down regulation mechanisms and does not exclude the possibility that RCMV is able to directly pull MHC II from the cell surface, irrespective of host cell recycling pathways. With that said, the greater effect seen in immature DCs does preferentially, albeit not exclusively, support a role for host cell pathways in RCMV induced down regulation of MHC II.

One possible mechanism for RCMV enhancement of MHC II recycling is the induced upregulation of the cellular E3 ubiquitin ligase, MARCH-I. Alternatively, the virus may encode its own previously unrecognized homologue to MARCH-I. Past reports have detailed the existence and function of virally encoded ubiquitin ligases in down regulation of surface molecules involved in antigen presentation and adhesion including MHC I (20, 43, 74). RCMV may also induce MHC II endocytosis in an Ub-independent fashion, manipulating the clathrin and dynamin dependent pathway.

Alternatively, the process may not involve recycling, but instead could be a virally encoded acid dependent protease, or specific degradation of a chaperone, such as HLA-DM that leads to the destabilization of MHC II at lysosomal pH (135). RCMV could interfere with the Ii signal sequence and retarget MHC II to the lysosome without the HLA-DM chaperone or peptide loading to stabilize it.

Whether the induced depletion is due to the removal of mature MHC II from the plasma membrane or not, the blockade of RCMV induced depletion of MHC II by the lysosomal acidification inhibitor, bafilomycin, suggests a lysosome/late endosome/pH dependent protease is involved, either virally encoded or host cell derived. The use of specific classes of protease inhibitors in combination with large scale viral deletion mutants will narrow the search.

Additionally, the mainstay of the Rat CMV model is its utility in studying the effects of CMV in transplant graft rejection. Recent reports have highlighted the role of DCs expressing MHC II in the development of regulatory T cells which mediate graft tolerance (183). The data presented in our study lays the groundwork for further exploration of the possible role of CMV manipulation of MHC II in DC in CMV induced graft rejection.

CHAPTER THREE: DISSECTING THE ROLES OF SPECIFIC CELL TYPES IN THE VIRAL LIFE CYCLE

Requirements for the Establishment of Persistence

The data presented in this Chapter outline the development of two models of *in vivo* infection designed to address questions related to the cellular and viral requirements for the establishment of CMV latency and persistence. The study presented in section A details a system to identify and isolate infected cells (of the peripheral blood and bone marrow, two sites believed to play critical roles in CMV persistence and latency) through the use of an RCMV mutant virus expressing GFP. Section B summarizes an alternative approach to the use of deletion mutant virus in ascertaining the roles of specific cell types in the pathogenesis, persistence and establishment of latency *in vivo*; this model utilizes the Cre-Lox recombination system to knock out viral replication in Cre expressing macrophages or endothelial cells.

A. CHARACTERIZATION OF RCMV INFECTED CELLS IN THE PERIPHERAL BLOOD AND BONE MARROW

In recent years several labs have used microarray analysis to study global transcription patterns in cells infected with HCMV and HSV *in vitro* as a model of viral latency *in vivo* (37, 78, 79, 264). However, a study published in 2007 demonstrated that viral transcription patterns *in vitro* do not reflect the more restricted transcription profiles observed *in vivo* (268). In addition to noting a stark divergence in patterns seen in cell culture versus those observed *in vivo*, Streblow *et al.* also recognized tissue specific viral transcriptional profiles. Whether the tissue specific profiles truly represent global tissue specificity or

rather a collection of variable cell types each with their own unique profile within the organ has yet to be determined. In an effort to delineate between the two possibilities studies are underway to isolate *in vivo* infected cells for analysis of gene expression profiles at the single cell level.

1. RESULTS

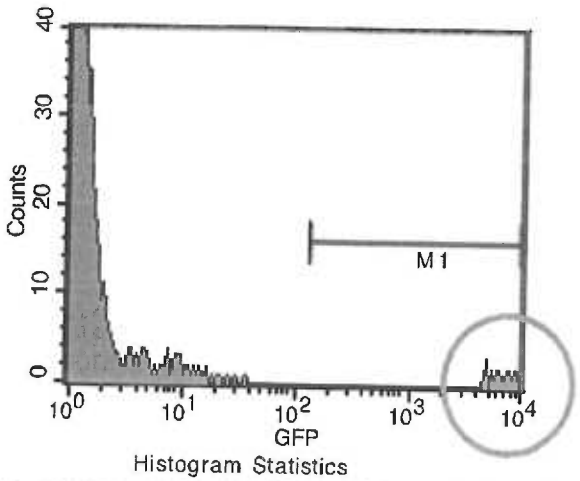
In vivo labeling of infected cells was achieved through the use of an RCMV mutant virus expressing GFP (as described previously) under the control of the eF1α promoter. Immune suppressed rats were infected with RCMV-GFP and harvested at 3, 5, 7 and 10 days post infection to monitor for peak GFP expression. GFP expression in γ-irradiated rats infected IP with $1x10^5$ PFU of RCMV-GFP was monitored in the solid organs via fluorescence microscopy (Figure 15). GFP expression in CSA-treated rats infected with $5x10^5$ PFU was tracked by flow cytometry analysis of Ficoll purified peripheral blood (PB) and bone marrow (BM) (Figure 16). GFP expression in the solid organs as well as the PB and BM peaked at 5 days post infection. At the peak of GFP expression PBMCs expressing high levels of GFP represented 0.077% of the total PBMC population and 0.006% of the total BMC population.

While purification over Ficoll resulted in a clean and rapid isolation of PB cells, the enrichment of lymphocytes, B cells, T cells and monocytes and a concomitant loss of polymorphonuclear cells (including granulocytes, neutrophils and eosinophils) lead to the use of Percoll gradients as an alternative means of

ORGAN	Day 3	Day 5	Day 7	Day 10	Day 21	Uninfected
SMG	-	+	+	++	++	-
HEART	+	+/-	+/-	+	-	-
LUNG	++	++	+	++	+	-
SPLEEN	++	++	++	++	-	-
LIVER	+/-	++	+	+	-	-
KIDNEY	+/-	++	+	+/-	-	-

FIGURE 15. Detection of RCMV-GFP in Infected Rat Tissues

Tissues harvested from RCMV-GFP infected rats at the indicated days post infection and were sectioned and analysed by fluorescent microscopy. Five sections were prepared for each tissue. Groupings: no GFP+ foci in more than 5 tissue sections (-); 1-5 GFP+ foci per 5 tissue sections (+/-); multiple foci per tissue section (+); and multiple foci per 20x microscope field (++).



Histogram Statistics

Marker	Events	%Gated	%Total	Mean	CV	Median
All	55260	100.00	100.00	6.69	3035.61	1.00
M1	45	0.0	0.08	6937.46	23.07	6978.31

PERIPHERAL BLOOD MONONUCLEAR CELLS

DPI	*total # events*	*# GFP hi pos*	*% total*
3	500,000	105	0.021
5	500,000	383	0.077
7	500,000	136	0.027
10	500,000	2	0.000

BONE MARROW

DPI	*total # events*	*# GFP hi pos*	*% total*	*# GFP lo pos*	*% total*
3	460,371	22	0.005	2410.00	0.523
5	461,718	26	0.006	1976.00	0.428
7	500,000	2	0.000	3946.00	0.789
10	500,000	0	0.000	249.00	0.050

FIGURE 16. RCMV-GFP Expression in Peripheral Blood Mononuclear Cells and Bone Marrow Cells

Rats were CsA treated 24h prior to infection, and harvested at the indicated days post infection. Peripheral blood and bone marrow cells were isolated by Ficoll density gradient centrifugation and analysed by flow cytometry for GFP expression. The percent of total PBMC and BMC positive for GFP is shown.

purification. Percoll step gradients yield a wider variety of purified cell types, allowing the recovery of PMN along with B cells, T cells and monocytes. Utilization of the Percoll density gradient centrifugation in combination with flow cytometry analysis using a variety of antibodies to various cell surface markers (including those specific for ECs, B cells, T cells, monocytes/macrophages/DCs, NKs, stem cells) lead to the identification of GFP positive cells that were also positive for granulocyte or CD11b/c markers, most likely monocytes, both of which have previously been identified as target cells *in vivo* (18, 218, 233, 286).

2. FUTURE DIRECTIONS

CHARACTERIZATION OF RCMV INFECTION OF CELLS OF THE PERIPHERAL BLOOD AND BONE MARROW

Although monocytes and granulocytes have both been previously identified as target cells *in vivo* the ability to label the cells by GFP expression provides two key advantages to current strategies identifying infected cells. One, GFP expression driven by a cellular promoter, eliminates the need for specific antibodies to viral proteins which either may or may not be expressed (or expressed at levels below detection) in individual cell types *in vivo*. Two, use of GFP expression to label infected cells, provides an effective way to universally isolate infected cells via Fluorescence Activated Cell Sorting (FACS). Transcriptional analysis of dual stained populations (GFP and specific cell markers) will facilitate the characterization of transcriptional profiles of infected subpopulations within the peripheral blood and bone marrow. While cells

infected *in vivo* with the RCMV-GFP virus can be isolated via FACS, the low number of cells which highly express GFP coupled with reduced live recovery of those cells after FACS is prohibitive to functional assays. However, there is a much larger population of cells (0.4% of the total BMC) which express a lower level of GFP. Whether or not the reduced level of GFP expression is an indicator of an abortive infection, or is due to low level fluorescence caused by the phagocytosis of GFP virus (as opposed to virus which has directly infected the cell and made it to the nucleus) remains to be determined. Likewise, it is presumptive to assume that elevated levels of GFP are indicative of a productive infection. Plaque assays and electron microscopy in combination with a more exhaustive identification of the cell types infected using multiple cell surface markers to more accurately phenotype the GFP positive cells would all aid in the characterization of both low and high GFP expressing cells.

B. DEVELOPMENT OF A CELL TYPE SPECIFIC REPLICATION KNOCK OUT VIRUS

In an effort to elucidate the roles of macrophage and endothelial cell tropism in the pathogenesis and persistence of CMV *in vivo*, a cell type specific replication knock out virus model system was developed utilizing the Cre-Lox system. In the past questions addressing viral tropism have utilized viral deletion mutants in which genes recognized as tropism determinants have been removed from the viral genome. Specifically studies investigating the role of macrophage infection in the lifecycle of CMV *in vivo*, have employed deletion mutant viruses in which

ORFs identified as macrophage tropism determinants have been removed or alternatively have utilized chemical depletion of macrophages. While deletion of ORFs recognized as tropism determinants can severely debilitate viral replication in macrophages *in vitro*, use of deletion mutants *in vivo* poses several major caveats to such studies. First, identification of all ORFs facilitating replication in a particular cell type is cumbersome at best, making a true cell type specific viral deletion mutant difficult to attain. Studies investigating macrophage tropism determinants have demonstrated varying degrees of replication knock down depending on the origin of the macrophage. Second, and perhaps more importantly, one cannot exclude the possibility that a deleted tropism ORF, may have an unintended phenotype. Gene products which function as tropism determinants in one cell type, may aid replication in another as yet undetermined cell type. Alternatively, deletion of a coding region for one gene product may disrupt regulatory sequences for another ORF. These disturbances in the viral genome can be difficult to recognize *in vitro*, but can cause major growth defects *in vivo*. Therefore an observed phenotype attributed to a disruption of macrophage tropism, may in fact be the result of an unrelated disturbance in the viral genome.

To combat the pitfalls of the deletion mutant approach, a novel alternative to the use of deletion mutant virus was developed to answer questions related to the role of CMV tropism for specific cell types *in vivo*. Utilization of the Cre-Lox recombinase system facilitated the development of a cell type specific replication

knock out virus capable of normal replication in non-Cre expressing cells, but inactivated in Cre-expressing cells. Cre recombinase catalyzes the recombination of two Lox P sites; when Lox P sequences are oriented in a direct repeat, recombination of the sites results in an excision of the intervening sequence (141). We designed a series of MCMV mutant viruses containing Lox P sites flanking (floxed) essential regions of the viral genome to be used in conjunction with transgenic mice expressing Cre under cell type specific promoters. The Cre transgenic mice used in these studies are C57Bl6 strain mice engineered to express Cre under the cell type specific promoters LysM (macrophage specific promoter) or Tie2 (endothelial specific promoter).

A recent study similarly utilized a Cre-Lox approach to explore questions of MCMV cellular tropism (228). However, the engineered virus and the questions asked are fundamentally different from the system we have described above. The Koszinowski group designed an MCMV recombinant with a GFP cassette containing a floxed premature stop codon replacing the m157 ORF. Expression of GFP from that recombinant virus can only be achieved once the virus completes a replication cycle in a Cre-expressing cell and the Lox sites recombine, excising the stop codon. The levels of green PFU to total PFU can then be compared to determine a relative contribution of each Cre expressing cell type tested to total virus production. However, once GFP expression is activated due to genomic rearrangement, all subsequent replication cycles will yield green virus, regardless of what cell type the virus replicates in. This results

in a skewed assessment of the contribution of each Cre expressing cell type to total virus production. Despite that caveat, the study produced a very interesting and unanticipated result. Sacher *et al.* intravenously infected transgenic mice expressing Cre under a hepatocyte specific promoter and tracked secondary viral dissemination from hepatocytes *in vivo*. Although infection of hepatocytes contributes greatly to the total viral load at early times post infection, virus originating from infected hepatocytes did not spread beyond the liver regardless of infection route.

In contrast to the Sacher study where viral genomic rearrangement marked virus as having replicated in the cell type in question at some point, our experimental design seeks to specifically knock out viral replication in Cre expressing cells. By specifically eliminating replication in a single cell type, while maintaining the overall integrity of the viral genome, each cell type's contribution to viral dissemination, replication and the establishment of latency/persistence can be more definitively dissected.

1. RESULTS

The Lox P mutant viruses engineered in the Nelson lab utilized the MCMV Bacterial Artificial Chromosome clone, pSM3-fr (293). Lox P sites were inserted into the MCMV BAC via homologous recombination. The first generation Lox P virus, clone I5, contains a Lox P site (and adjacent FRT sequence) inserted at nucleotide position 182,361 within the intron separating exon 1 and exon 2 of the

Immediate Early 1/3 gene, for a total 121 base pair insertion. An additional Lox P site with adjacent FRT flanked Kanamycin resistence cassette at nucleotide position 79,563 within the intergenic region separating M53 and M54 was inserted into the pSM3-fr containing the Lox P site in the IE1 region, for an additional 1.5kb insertion. Infection of a Cre-expressing cell results in deletion of 100kb of intervening sequence between the Lox P sites including the essential ORFs encoding the DNA polymerase, gB and IE1/3 proteins.

The second generation Lox P virus, clone V7, was designed with the goal of increasing the efficiency of the Cre-recombinase by both bringing the Lox P sites closer together within the viral genome and within a region that should be immediately transcriptionally active, thus in an open conformation and available to the recombinase. Lox P sites were positioned to excise a <7kb region encompassing nucleotides 175,479 to 182,361. Excision of intervening sequence removes m121 to the essential IE1/3 ORF. Growth curves in fibroblasts revealed no advantage of this clone over the previous 100kb Lox clone, I5, in terms of recombination efficiency as indirectly measured by viral titer in Cre-expressing NIH3T3s.

The third generation Lox P virus, clone W6, was generated to combat innate resistance to MCMV infection inherent to the C57Bl6 strain utilized in the study. In 2003 Voigt *et al.* identified the m157 ORF gene product as the ligand for the

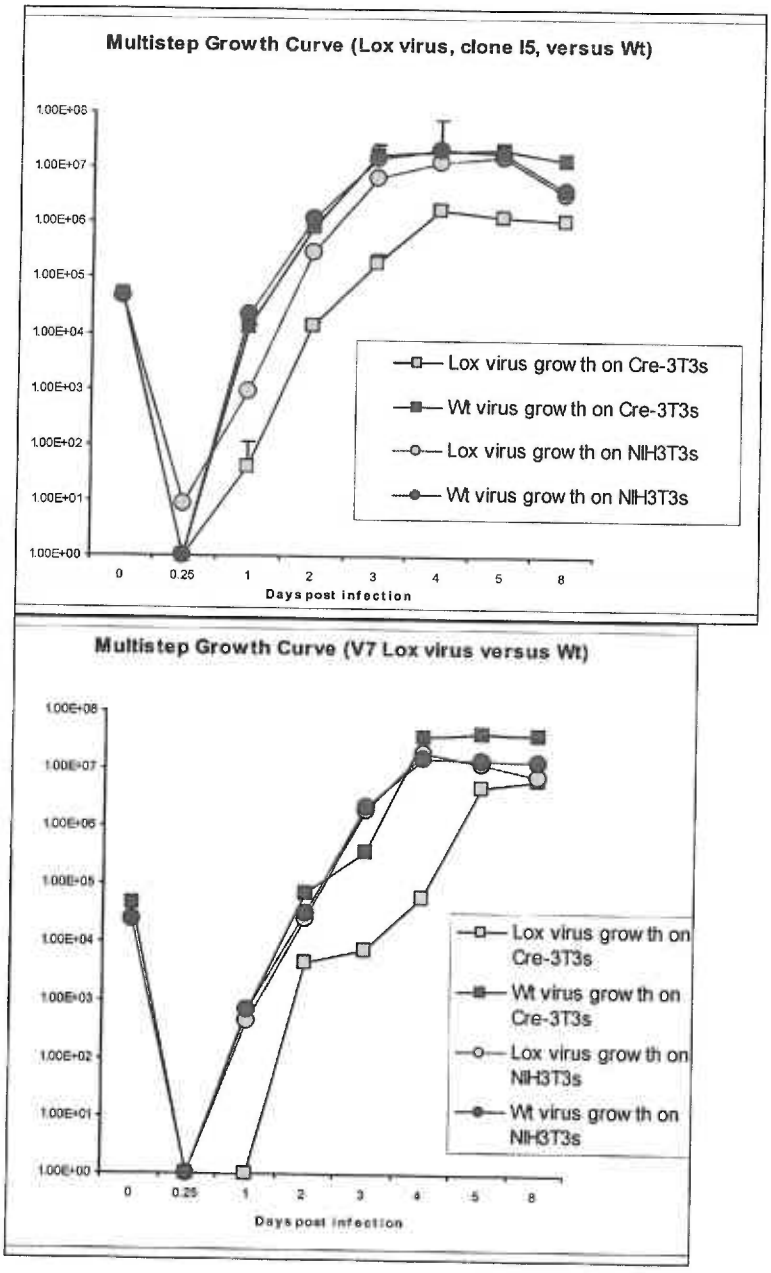


Figure 17. Lox Virus Growth in NIH3T3 and Cre-3T3

NIH 3T3s and Cre-3T3s were infected at an MOI equal to 0.1 with sorbitol pelleted MCMV Wt Smith strain, the I5 Lox clone (100kb) or the V7 Lox clone (7kb), with each condition in triplicate. Supernatants from infected cells were harvested at the indicated times post infection. Viral titers were determined by duplicate plaque assay on NIH3T3s. Y axis: Viral titers PFU/ml

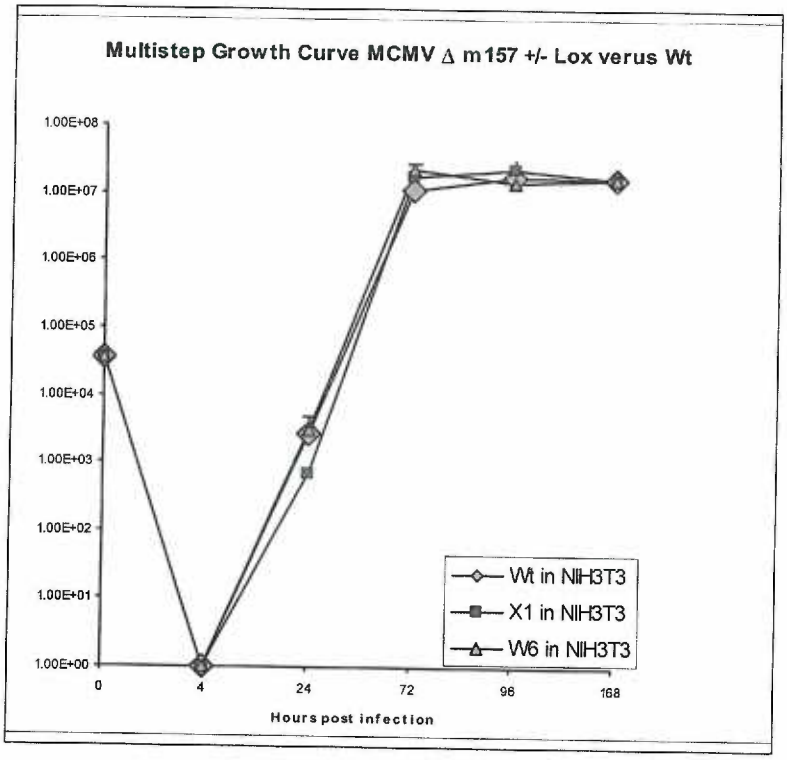


Figure 18. Δm157 Mutation (with and without Lox mutation) Multistep Growth in NIH3T3

NIH 3T3s were infected at an MOI equal to 0.1 with sorbitol pelleted MCMV Wt Smith strain, the W6 Δm157 Lox clone, or the X1 Δm157 clone, with each condition in triplicate. Supernatants from infected cells were harvested at the indicated times post infection. Viral titers were determined by duplicate plaque assay on NIH3T3s. Y axis: Viral titers PFU/ml

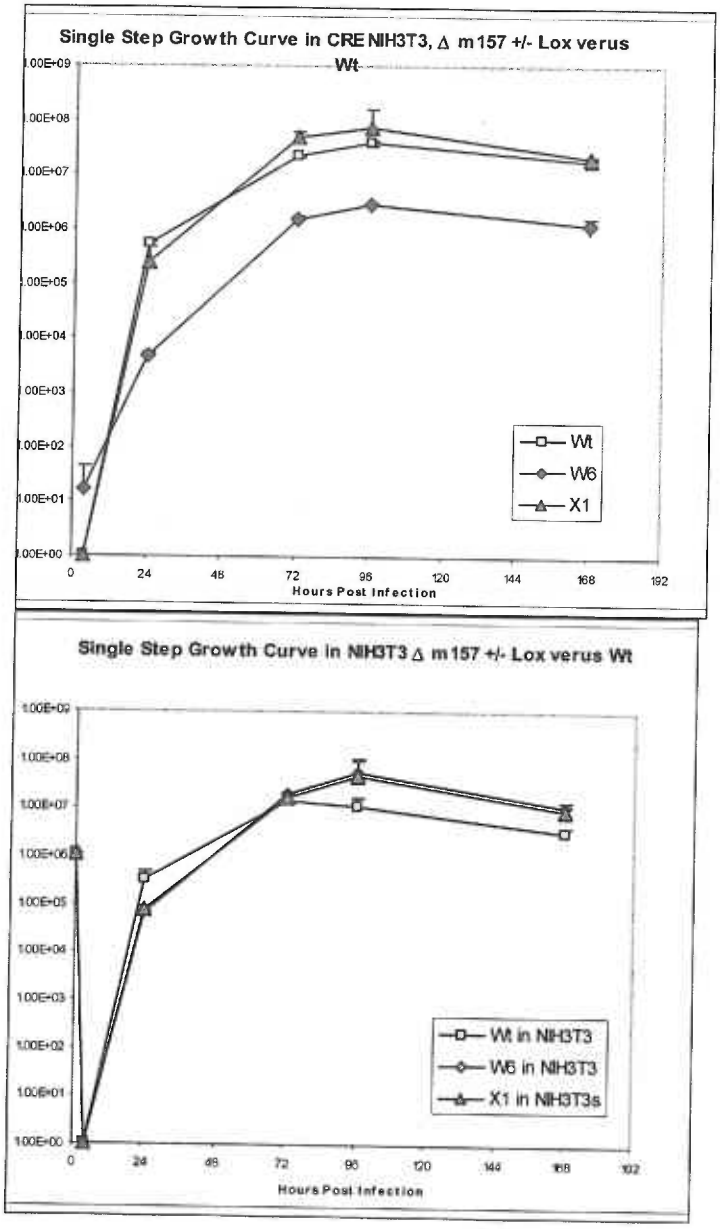


Figure 19. Δm157 Mutation (with and without Lox mutation) Single Step Growth in NIH3T3 and Cre-3T3

NIH 3T3s were infected at an MOI equal to 3.0 with sorbitol pelleted MCMV Wt Smith strain, the W6 Δm157 Lox clone, or the X1 Δm157 clone, with each condition in triplicate. Supernatants from infected cells were harvested at the indicated times post infection. Viral titers were determined by duplicate plaque assay on NIH3T3s. Y axis: Viral titers PFU/ml

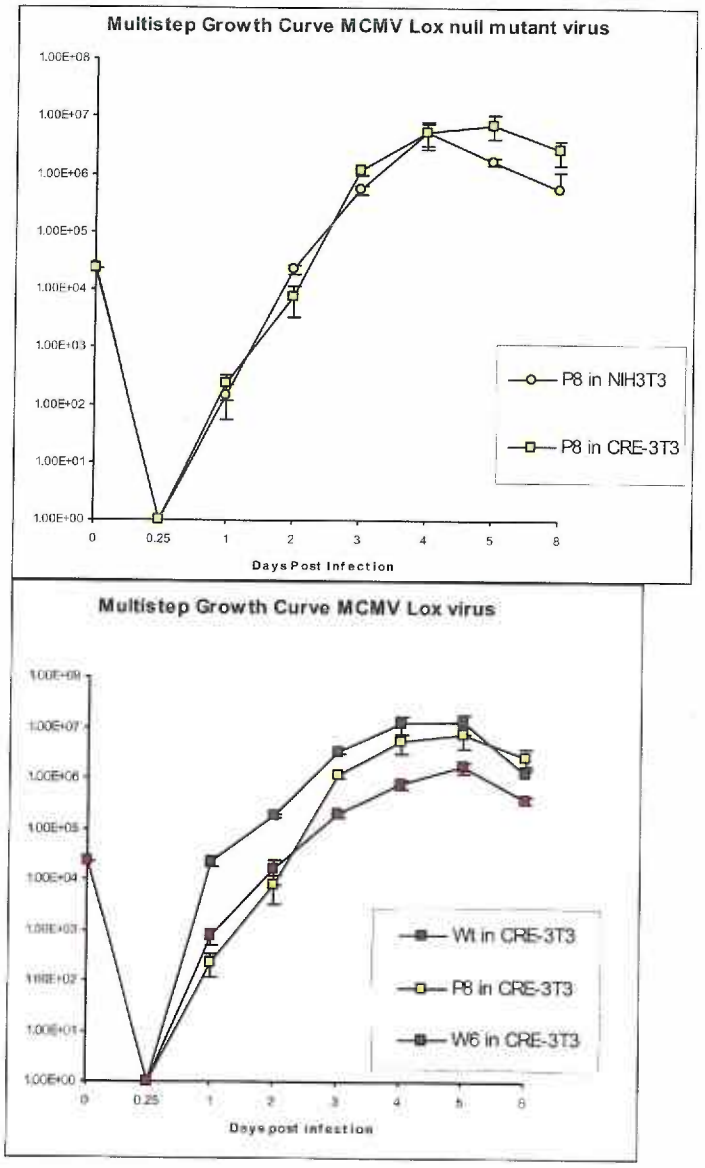


Figure 20. Multistep Growth in NIH3T3 and Cre-3T3, Wt versus Lox Null versus Δm157 Lox clone

NIH 3T3s were infected at an MOI equal to 0.1 with sorbitol pelleted MCMV Wt Smith strain, the W6 Δm157 Lox clone, or the P8 Lox Null clone, with each condition in triplicate. Supernatants from infected cells were harvested at the indicated times post infection. Viral titers were determined by duplicate plaque assay on NIH3T3s. Y axis: Viral titers PFU/ml

NK cell activating receptor Ly49H, which mediates NK cell resistance observed in the Ly49H+ C57Bl6 strain, and absent in the MCMV susceptible Ly49H- BalbC strain (292). Clone W6 utilized the V7 clone as the genomic backbone and replaced the m157 ORF (deleting sequence between nucleotides 216,250 and 216,885) with an Ampicillin resistance cassette, thus maintaining the 7kb LoxP sites with the additional deletion of the m157 ORF. While the deletion of the m157 ORF counters the claim of an intact viral genome to study cellular tropism, it may be a necessary caveat to study MCMV tropism in innately resistant mouse strains.

An MCMV clone, P8, with mutated Lox P sites not recognized by the Cre-recombinase, was generated to control for the possible disruption of regulatory sequences by insertion of the Lox P sites into the IE1/3 and gB/pol intergenic regions. *In vitro* growth curve analysis confirmed wildtype-like replication in both Cre-expressing and wildtype NIH3T3s.

Single insertion of each Lox P site into the viral genome was confirmed via Southern Blot analysis of restriction digests. Multiple restriction digests and ethidium bromide gel analysis confirmed a lack of gross genomic rearrangements. Single and multistep growth curves were performed in wildtype NIH3T3 mouse fibroblasts and a stable Cre-expressing NIH3T3 cell line developed in the lab. Recombination in Cre-expressing NIH3T3 cells was confirmed via polymerase chain reaction.

While all of the Lox viruses (excluding the mutated Lox site virus P8) exhibited a 1-2 log (90-99%) reduction in viral titers in Cre-expressing fibroblasts as compared to non-Cre expressing fibroblasts, replication was not fully eliminated *in vitro*. However, a complete knock out *in vitro* may be a lofty goal for this system, highly dependent upon the efficiency of the CRE under specific cellular promoters and maintenance of the Lox sites in the virus. While the Koszinowski system only requires one recombination event before the GFP virus is amplified, our system requires repeated fidelity of the CRE recombinase to maintain the lox virus replication knock out. The fact that the Lox viruses were still able to replicate in fibroblasts (albeit at 1-10% of wildtype levels) may be in part be due to the fact that these cells are highly permissive to CMV. However, even a slight growth defect in fibroblasts, may completely eliminate replication in a less susceptible cell type, such as the macrophage and endothelial cell. As well, differences in promoter activity and the copy number of transgenes inserted into the mouse genome, all point to the need for confirmation *in vivo*.

The first round of *in vivo* studies utilized the viral clones on hand at the time, I5 (the 100kb Lox virus) and Wt (BAC derived Smith strain). LysM Cre and wildtype C57Bl6 mice were infected with Wt MCMV or the I5 clone via the intraperitoneal route. Harvested organs were analyzed by quantitative Real Time PCR to determine genome copy number per μg of total DNA. While a great deal of variability between animals is evident and the low number of replicates for each treatment complicates a definitive interpretation of the data at this time, an

apparent trend does emerge. Viral genome copy numbers (in the spleen, liver and kidneys, *but not in the salivary gland*) were elevated at days 3 and 5 in LysM-Cre mice infected with the Lox virus as compared to those infected with the Wt virus. However due to the high degree of variability observed in the C57Bl6 control mice, it is not clear if the trend seen in the LysM-Cre mice is due to the Cre-mediated inactivation of the Lox virus, or perhaps disruption of a regulatory sequence in the Lox virus.

2. FUTURE DIRECTIONS

CELL TYPE SPECIFIC VIRAL REPLICATION KNOCK OUT

Utilization of the Lox virus with mutated inactive Lox sites, clone P8, will be a better control for the possibility of disruption of intergenic regulatory sequences due to insertion of the Lox P sites. Challenging the mice with a higher dose of virus may also alleviate the degree of variability observed between animals by establishing a more robust acute infection, thereby increasing viral replication and moving the baseline of the data further from the limit of detection. Alternatively, use of the m157 KO mutants would reduce the innate resistance to MCMV infection characteristic of the C57Bl6 strain and absent in the BalbC strain more commonly used for *in vivo* MCMV studies. Finally, there is a degree of variability that is inherent in all animal studies, and as such a larger sample size (minimum of five) is required to attain statistical significance.

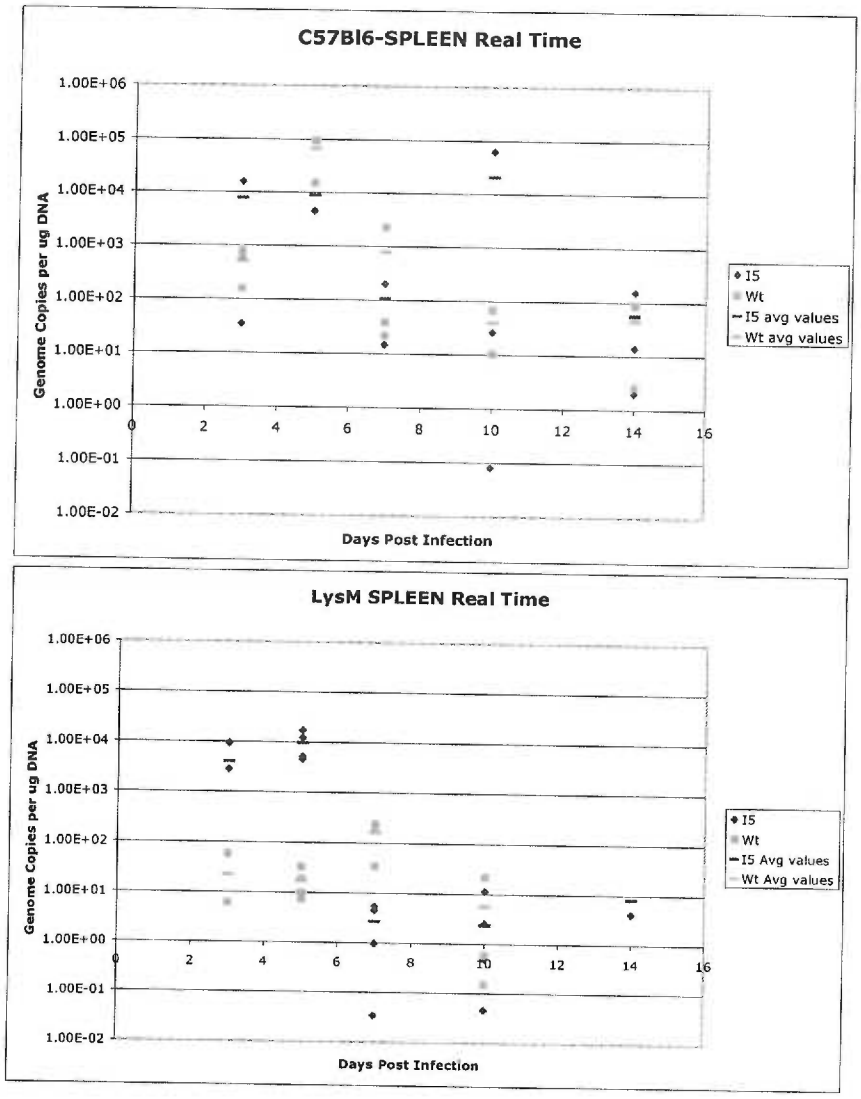


Figure 21. Lox Virus and Wt Replication in Spleen of LysM Cre and Wt C57Bl6 mice

C57Bl6 and C57Bl6-LysM Cre mice were infected at 4-6 weeks of age via intraperitoneal (IP) route with $1x10^6$ PFU of I5 (100kb Lox virus) or Wt MCMV and then sacrificed at the indicated days post infection. Harvested organs were individually weighed and 10% tissue homogenates were prepared by dounce homogenization and frozen at -80C before viral genome copy number was ascertained by quantitative Real Time PCR.

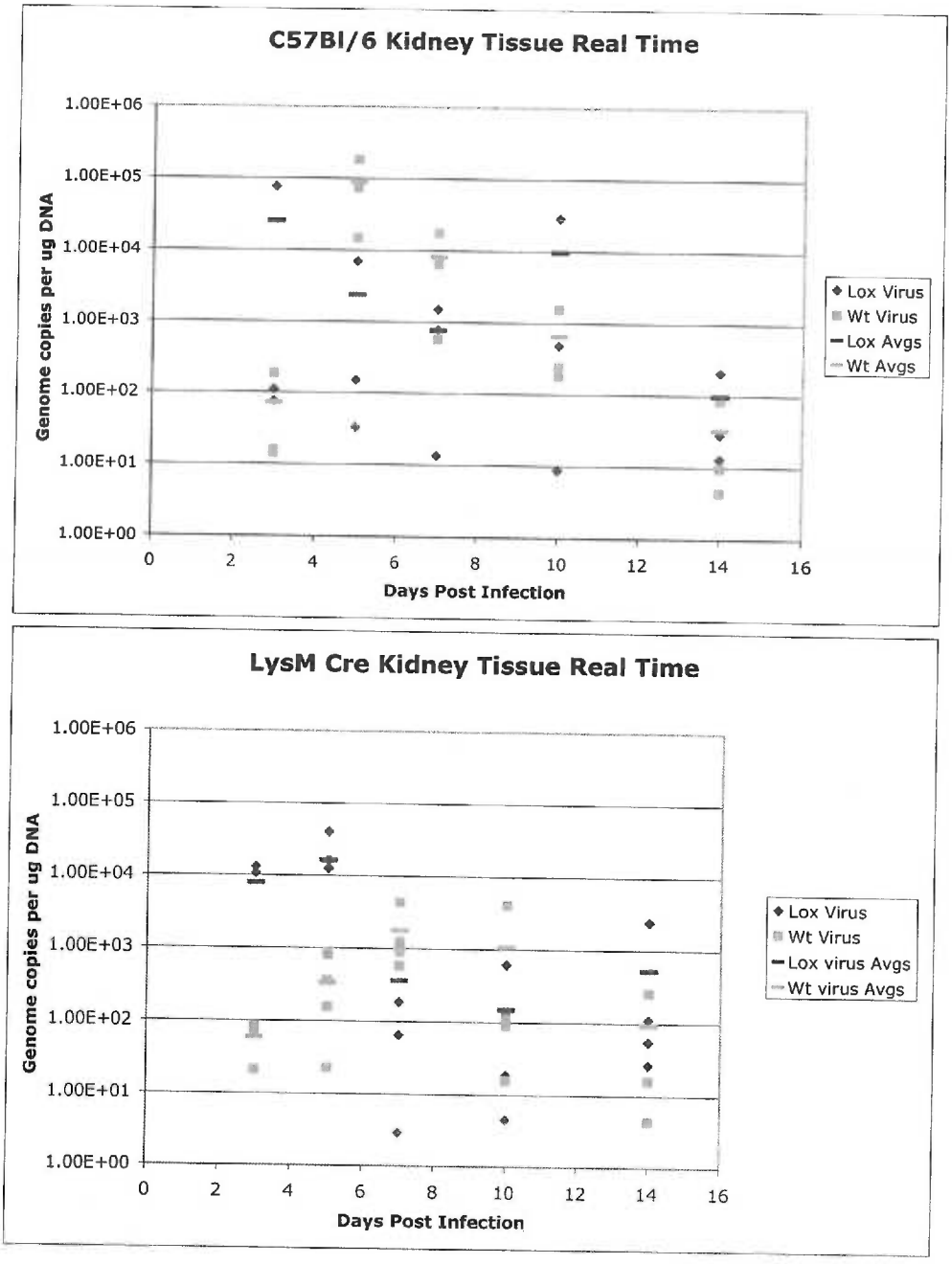
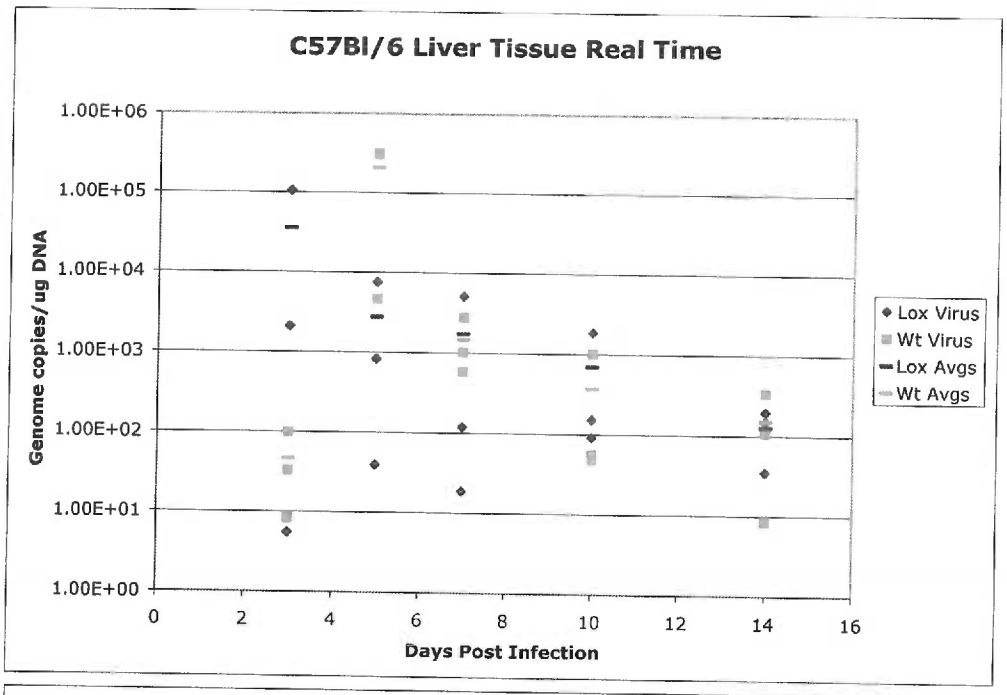


Figure 22. Lox Virus and Wt Replication in Kidney of LysM Cre and Wt C57Bl6 mice

Experiment performed as described in Figure 21.



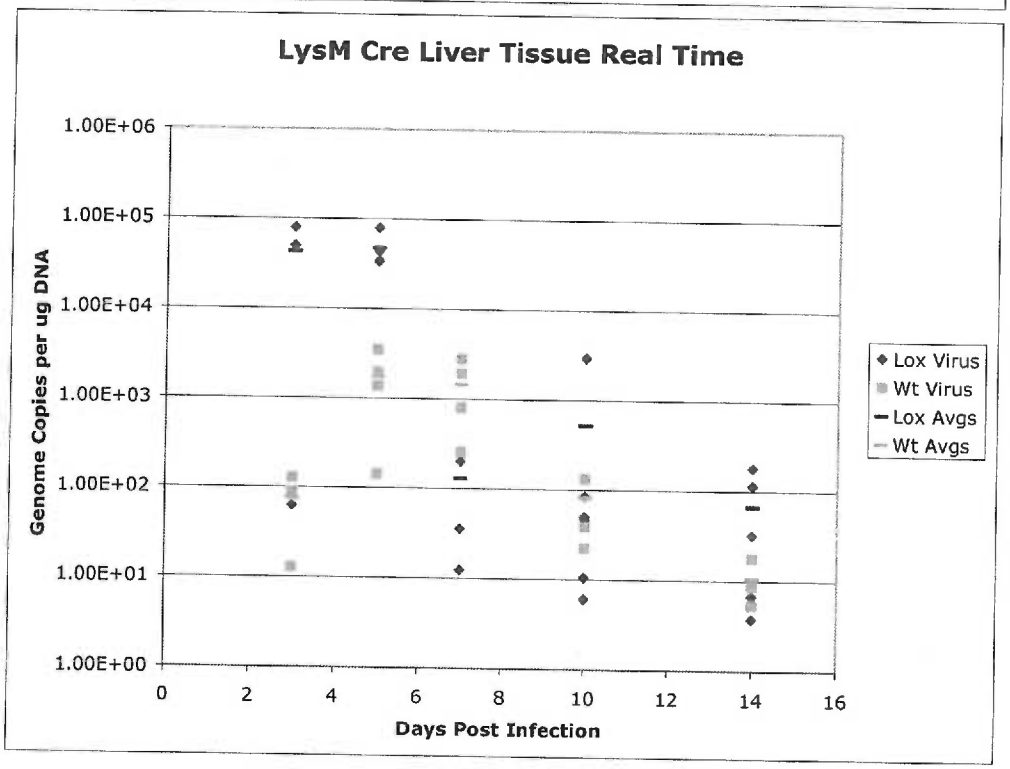
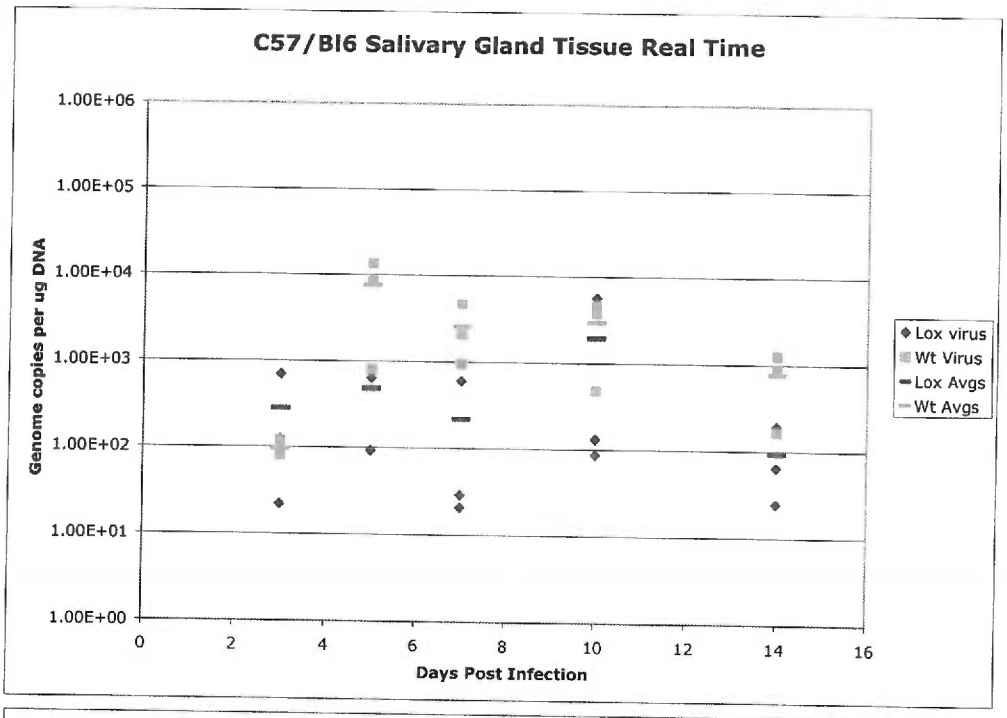


Figure 23. Lox Virus and Wt Replication in Liver of LysM Cre and Wt C57Bl6 mice

Experiment performed as described in Figure 21.



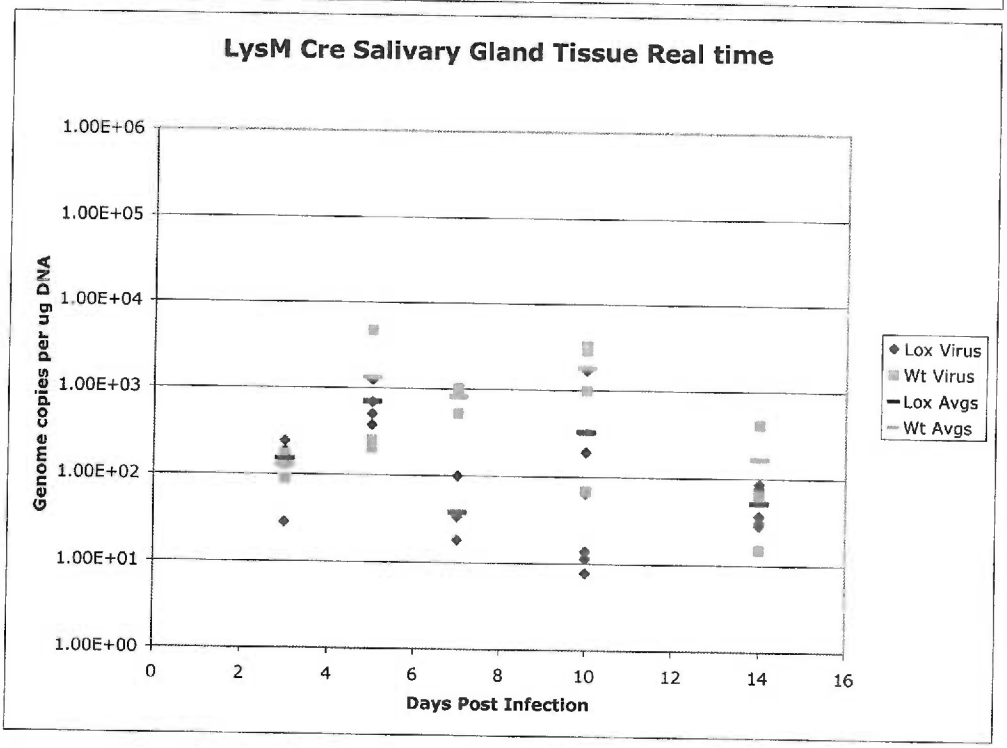


Figure 24. Lox Virus and Wt Replication in Salivary Gland of LysM Cre and Wt C57Bl6 mice

Experiment performed as described in Figure 21.

While the IP data does lend insight into the role of CMV infection of macrophages in the establishment of an acute infection, the infection route is not ideal for questions related to viral dissemination. Localized subcutaneous infection routes, such as foot pad inoculation, would be a better measure of each cell type's contribution to viral dissemination. As well, an analysis of the Lox virus within the Tie2 (Cre-EC) mice would be worthwhile, the efficiency of the CRE recombinase under a different promoter (at different copy number) may be more efficient at inactivating the Lox virus. Additionally the effect of the lox virus in Cre mice may be more pronounced in a latency/reactivation model. However, the possibility of escape mutants is a major caveat to long term infection studies and as such would need to be monitored.

Professional APCs play a unique role in the life cycle of CMV *in vivo*, as both mediators of front line defense against viral infection and as target cell types for infection. Tissue macrophages, like dendritic cells, act as sentries in the periphery. Detection of the viral pathogen triggers a cascade of antiviral effector functions which exhibit both innate and adaptive immune response characteristics. Elucidating the roles of MCMV replication in macrophages and ECs is critical to advancing our understanding of the mechanisms of both acute infection and long term CMV persistence and reactivation from a latent or persistent state. A complete understanding of the requirement for replication in macrophages and endothelial cells may also help to identify potential therapeutic strategies.

While a multitude of studies have sought to delineate the effects of CMV infection of macrophages and dendritic cells on their ability to carry out their antiviral effector functions *in vitro*, few have been able to address the impact of viral infection of macrophages on the cell's antiviral effector function *in vivo*. The finding that virus which is replication defective in macrophages exhibits an early advantage in viral replication *in vivo* suggests that viral replication in macrophages may play a protective role thereby limiting viral replication in the organ. The timing of the increase in viral replication suggests a difference in the innate immune response.

CHAPTER FOUR: DISCUSSION

A. Rat Cytomegalovirus Mediated MHC II Depletion

The data presented in this thesis describe the development of a highly effective system to study RCMV-mediated depletion of MHC II in bone marrow derived dendritic cells. Both immature and mature BMDCs were found to be highly permissive to RCMV infection. The infection efficiencies of immature BMDCs was similar to that found in permissive fibroblasts. Cell surface expression and intracellular stores of MHC II were reduced by greater than 90% 48hpi, despite the absence of homologues to US2, US3 or UL111A. The high degree of infection at low MOI coupled with the dramatic effect seen in steady state levels of MHC II permitted biochemical analysis in the relevant *infected* cell type. RCMV was found to mediate MHC II depletion by two mechanisms, one of which has not previously been described for any virus in the literature, induced lysosomal degradation of steady state MHC II. Pulse chase experiments in combination with inhibitor studies and the use of RT-PCR revealed a post-transcriptional and likely pre-translational effect of RCMV on nascent MHC II, an effect recently detailed in an elegant study examining RhCMV mediated down regulation of MHC I.

In addition to the three major findings detailed above (model system, lysosomal mediated degradation, post-transcriptional effect) five more characteristics of MHC II down regulation unique to Rat CMV were described in chapter two. This

is the first report to describe the down regulation of the HLA-DQ form of MHC II (known as RT1.D in rats) by any CMV. While previous reports have focused on down regulation of HLA-DR and HLA-DM and their homologues in mice, the study presented in the results section is the first to show a CMV induced depletion of HLA-DQ. This is also the first report to describe a lack of bystander effect in MHC II down regulation in CMV infected APCs. Reports for MCMV and HCMV have demonstrated that both can mediate the down regulation of MHC II in APCs through the action of secreted IL10 in the culture supernatants. The lack of bystander effect coupled with the inability of cultured supernatant to mediate the down regulation of MHC II (RT1.B) argues against a role for IL10 or other secreted effectors to mediate the depletion of RT1.B at the cell surface. Rather than an RCMV or cell type specific phenomena, more recent data (demonstrating a partial down regulation of the HLA-DR homologue in GFP negative cells) points to a difference in the regulation of HLA-DQ versus HLA-DR, again, not previously reported in the literature. Virion structural components have also been implicated in HCMV mediated MHC II down regulation in macrophages infected with UV-inactivated virus. This observation was inconclusively linked to pp65, and while RCMV possesses a pp65 homologue, UV-inactivated virus failed to mediate the down regulation of MHC II in BMDC, but rather required replication competent virus. Finally, proteasome mediated degradation of nascent MHC II alpha was not observed. This last finding was surprising, given the report by Tomazin *et al.*, where expression of US2 was found to lead to the proteasome-dependent degradation of HLA-DRα. Although RCMV does not encode a US2 homologue,

there are many examples within the CMV field where convergent evolution has lead to the acquisition of ORFs with vastly different sequences whose encoded proteins, produce the same effect in the cell.

By far, the most intriguing finding of Chapter 2 is the identification of viral-induced lysosomal mediated degradation of MHC II. Although use of the inhibitor bafilomycin, clearly shows that RCMV induced depletion of MHC II is dependent upon acidification of the lysosome, the question remains as to which point(s) in the pathway is RCMV inducing the loss of MHC II.

Beyond a lysosomal mediated mechanism, the other unique feature of this study is an exploration of mechanism in the *infected relevant cell type*. The Rat CMV bone marrow derived dendritic cell system is unique in its capacity to explore questions of biochemical mechanism. Because of the exquisite sensitivity of rat BMDCs to infection with CMV, coupled with the near complete loss of steady state levels of MHC II, biochemical analysis of CMV mechanisms of MHC II depletion can be explored in the context of DC infection. Although a multitude of studies have clearly identified macrophages and dendritic cells as important HCMV target cell types *in vivo*, efficient infection of professional APCs *in vitro* has remained elusive. Macrophages and dendritic cells are dynamic cell types, with variable phenotype and function dependent on culture environment. As such, the ability to replicate the uniquely susceptible cell type and culture system *in vitro* is a continuing challenge to the study of HCMV infection of professional APCs.

Furthermore, the importance of direct infection models and the limitation of transfection based studies are highlighted when reviewing the literature on US2 and US3 mediated MHC II down regulation. Due to limitations in infection efficiency, early studies investigating HCMV-induced MHC II modulation were executed using an astrocytoma cell line transfected with the MIITC transactivator. The gene products encoded by US2 and US3 were found to modulate MHC II expression in U373-MIITC cells transfected with the US2 or US3 ORFs. While the identification of ORFs directly modulating MHC II expression was a profound advancement in our understanding of CMV subversion of antigen presentation, and to date no one has reported a more detailed account of the mechanisms of CMV induced MHC II depletion, there is still some debate in the literature as to the efficacy of US2 and US3 in professional APCs.

One study briefly describes an experiment in which PBMC derived *in vitro* differentiated DCs were infected with a replication incompetent adenoviral expression vector containing the US2 ORF (214). One time point was examined and no discernable effect was seen on cell surface HLA-DR expression. Much has been made of this one experiment. While the study contributes to our understanding of the relationship between US2 and MHC, all that can be concluded from this one figure is that US2 expression alone is not sufficient to affect HLA-DR surface expression on PBMC derived DC at that time point. It does not conclusively dismiss the multiple publications addressing US2 down regulation of MHC II (in U373 cells, epithelial cells, glial cells and DCs). It does

not exclude the probability of multiple, redundant ORFs mediating MHC II down regulation. Nor does it negate the effectiveness of US2 mediated down regulation of MHC II in other CMV target cells (99). Although the Rehm study utilized DCs, it was not in the context of HCMV infection, but rather adenoviral infection. Use of infection based systems permit one to examine the ORFs in question without the confounding factors of protein expression levels, toxicity or stability, due to lack of normal binding partners. Additionally, cell type specific differences in MHC II presentation pathways may play a role in the efficiency of CMV-induced MHC II depletion. CMV can infect a variety of cell types which upon stimulation can naturally be induced to express MHC II. Hegde *et al.* explored the possibility of a cell type specific function of US2-mediated MHC II down regulation and found that epithelial and glial cells were both susceptible to US2 induced MHC II depletion (99). Finally, it is likely that multiple ORFs mediate the down regulation of MHC II. Using the U373-MIITC system Cebulla, *et al.* directly infected the cell line with wildtype HCMV or a deletion mutant, RV7186, lacking the IRS1-US11 region and found that the mutant lacking US2 and 3 was still able to down regulate MHC II but only at nearly two thirds capacity, suggesting both the augmenting effect of US2/3 on MHC II down regulation and the presence of other as yet unidentified ORFs modulating MHC II.

The paucity of *in vitro* models to directly study HCMV infection of primary APCs has lead to the use of a variety of substitute models to investigate HCMV

mediated down regulation of MHC II. The sometimes incongruous data on HCMV induced depletion of MHC II is reflective of the variability of these systems.

B. Characterization of Rat Cytomegalovirus Infection of Bone Marrow and Peripheral Blood Cells *In Vivo*

CMV gene expression *in vitro* occurs in three kinetic phases, immediate early, early and late with more than 95% of the predicted ORFs expressed (268). However a detailed study examining Rat CMV transcription profiles found that those from tissues infected *in vivo* differed dramatically from *in vitro* profiles (268). Streblow and colleagues observed tissue specific patterns of viral gene transcription which differ dramatically from those patterns observed in fibroblasts, endothelial cells and smooth muscle cells cultured *in vitro* (268). The *in vivo* patterns were highly restricted, with just a fraction of the ORFs being expressed. In addition, the expression of some ORFs appears to be restricted to specific tissues. The observation of organ specific transcriptional profiles begs the question: is the profile due to a combination of multiple unique cell type specific transcriptional profiles? An understanding of cell type specific viral gene expression will likely lead to insights in predicting genes involved in viral immune evasion and pathogenesis.

Use of the GFP-expressing RCMV mutant virus described previously provided a means to label virally infected cells without the need for antibodies to specific

viral proteins whose levels may vary depending on cell type infected. Infection of γ-irradiated or CSA treated rats was in keeping with the immune suppression model of CMV infection. Peak levels of infection (as measured by GFP expression) for solid organs, bone marrow and peripheral blood were all found to occur at five days post infection. While the percent of total bone marrow and peripheral blood cells that highly expressed GFP was low (at less than 8 cells per 10,000 PBMC and 6 cells per 100,000 BMC) even at the peak of expression, the numbers were high enough for a limited evaluation of cell surface marker expression. Antibodies directed against specific cell surface markers for granulocytes, monocytes, T cells, B cells, ECs, NKs and stem cells were used to identify infected (GFP positive) cells by flow cytometry analysis. Two populations of GFP positive cells were identified, those which stained with an anti-granulocyte antibody and those that stained positively with an anti-CD11b/c antibody, likely monocytes. In confirmation of our findings, both populations of cells have been previously identified as CMV target cells *in vivo* (18, 218, 233, 286). The RCMV-GFP rat model provides a means to identify and possibly isolate cells infected *in vivo.* Isolation of individual infected populations within the peripheral blood and bone marrow may provide insight into the nature of CMV infection of prime targets implicated in dissemination, persistence, latency and immune control.

Labeling of infected cells with GFP under a universal cellular promoter should allow for an unbiased identification of infected cell types. Whereas previous

studies have relied on the identification of infected cells through the expression of ORFs highly transcribed *in vitro*, but as is now understood, restricted *in vivo*. Furthermore the use of GFP will facilitate Fluorescence Activated Cell Sorting (FACS) of infected cells for future transcriptional profile analysis. While transcriptional analysis is a powerful tool to advance our understanding of viral replication/persistence and mechanisms of immune evasion, it is just the beginning. The beauty of global transcriptional analysis is the vast number of questions it can spawn. For example, the finding that certain immune modulatory ORFs are expressed in some tissues and not others points to the possibility of tissue specific immune control (268).

C. Development of a Cell Type Specific Replication Knock Out Virus Model

Although monocyte derived macrophages (MDM) are believed to play a central role in HCMV persistence and dissemination in the host, little is known about the replication of HCMV in these cells. The vast majority of studies investigating the role of macrophage tropism in the viral life cycle have utilized MCMV/mouse model in combination with tropism determinant knock out viruses (93-95, 161). While these studies have suggested a dependence on replication in macrophages for viral dissemination and pathogenesis, analysis of the data obtained using deletion mutants is complicated by the limitations of the approach (94). First, viral genes that act as tropism determinants must already be identified, a process that is laborious. Second, deletion mutants are often

replication deficient in cell types not targeted for replication knock out (30, 162, 295, 296). A viral gene that is essential for replication in one cell type can prove to be augmenting in another. Third, it is extremely difficult to definitively prove that a gene involved in a cell type specific replication cycle is not functioning in another capacity in a different cell type. The compact nature of a viral genome, even one as large as CMV, predicts that any one gene product may have multiple functions.

To date, four macrophage tropism determinants have been identified in MCMV: ORFs m140, m141, M36 and M45 (93-95, 161). While these ORFs are homologues to the US22 gene family present in HCMV, sequence and positional homology between the two viruses has not been an exact predictor of functional homology (31, 86). Furthermore, studies have shown that while deletion of multiple macrophage determinants greatly limits MCMV's ability to replicate in macrophages, the deletion does not eliminate replication (94), suggesting either an additional tropism determinant with compensatory function or redundancy elsewhere in the genome, a common occurrence in herpesviruses.

Utilization of the Cre-Lox approach described in Chapter 3 provided a novel alternative to the use of deletion mutants in elucidating the role of specific cell types in the CMV life cycle. While the *in vivo* data suffered from a high degree of variability between individual animals and an insufficient number of replicates for each sample, one noticeable trend did emerge; the Lox virus had an early

replicative advantage in the spleen, liver and kidney of CRE-Mac mice as compared to wt virus. Repeat experiments in which the control C57Bl6 mice display less variability between the wt and Lox virus would need to be performed before definitive interpretation of the data in CRE-Mac mice. However, should the trend persist in better controlled repeat experiments, the data may indicate that CMV replication in macrophages provides an overall net protective effect for the host at early times post infection.

As a professional APC, the infected macrophage possesses a unique potential to secrete a combination of cytokines in response to CMV infection, those characteristic of both an infected cell and an immune surveillor. It is this combination of cytokines that may result in the production of a microenvironment better able to resist localized viral spread during the innate immune response to acute infection. The early times post infection that the Lox virus appears to have a replicative advantage would suggest an edge in evading the innate immune response. NK cells play a critical role controlling early stages of CMV infection and macrophages are known to modulate NK cell responses through the secretion of various cytokines.

IFNα, IFNβ, TNFα and IL12 activate NK cell cytolytic potential. IFNα and IFNβ are primarily produced by leukocytes and virally infected cells; both stimulate the upregulation of MHC I, shut down protein synthesis and enhance NK blastogenesis. Macrophages and B cells are the primary producers of IL12, a

widely recognized activator of NK cells, stimulating the production of IFNγ. HCMV infected macrophages have been demonstrated to secrete IL12, TNFα and IFNγ (246). Treatment of NK cells with TNFα and IL12 has a syngeristic effect inducing activation and IFNγ secretion by NKs. Heise and colleagues observed an increase in MCMV titers in the livers of IFNγ depleted SCID mice. Similarly, the roles of TNFα and IL12 were confirmed by Yerkovich *et al.*, whose *in vivo* administration of soluble receptor to TNFα and/or antibody to IL12 resulted in an increase in MCMV titers in the livers of BalbC and C57Bl6 mice. Finally, some of the most compelling evidence to suggest a link between CMV *infection* of macrophages (versus exposure to virus) and altered cytokine profiles *in vivo* was published by the Campbell lab in 1999 (96). The Campbell group observed a three to five fold induction in protein levels of IFNγ, TNFα and IL12 in the spleens of mice infected with wt or revertant virus as compared to the spleens of mice infected with the macrophage tropism knock out virus Δm139-141. This increase was only apparent in intact spleens; those mice whose spleens had been depleted of macrophages, had no increase in cytokine protein levels. Likewise, depletion of liver resident macrophages (Kupfer cells) correlated with increased viral titers in the liver.

With the recent characterization of virally encoded macrophage tropism determinants, one of which encodes a Cox-2 homologue, coupled with the availability of pharmacological inhibitors of Cox-2, replication of CMV in macrophages becomes an obvious and easy target for therapeutic drug

development. However, if replication of the virus in macrophages acts as a self-limiting stage to control acute infection and push the virus life cycle to a more subdued persistent or long lived latent infection, then by targeting replication in macrophages, the balance may tilt in favor of a more life threatening acute infection. A recent study published in 2003 examined the effects of selective inhibition of Cox-2 in MCMV-infected apoE mice (224). While the authors fully expected viral replication to be reduced and the anti-inflammatory activity of Cox-2 inhibitors to decrease the incidence and severity of atherosclerosis, the opposite effect was observed. Treatment of the mice with Cox-2 inhibitors resulted in an increase in both viral load and incidence of atherosclerotic lesion. This study highlights the importance of dissecting the protective versus pathological potential of CMV infection of macrophages and the use of animal models in determining outcomes *in vivo*.

ADDENDUM : SUPPLEMENTARY DATA

RCMV MEDIATED DEPLETION OF MHC II

The development of the Rat Cytomegalovirus bone marrow derived dendritic cell system coupled with the initial observation that RCMV is able to efficiently infect BMDC and dramatically impact MHC II surface expression, resulted in a number of interesting findings, each of which has the potential to be expanded into their own in depth study on the impact of RCMV infection of DC. Questions related to Rat CMV specific versus conserved CMV mechanisms of MHC II down regulation were explored. RCMV was found to deplete the rat homologues of HLA-DQ and HLA-DR by different means. In addition, the impact of RCMV infection on DCs of differing origin (peripheral blood mononuclear cell versus bone marrow derived *in vitro* differentiated DC) and varying states of maturation (LPS matured before and after infection) was examined. Herein, preliminary data pertaining to RCMV infection of DCs in the context of data currently present in the literature as well as a brief outline of potential experiments to expand these areas of study.

A. Susceptibility of Bone Marrow Derived Versus Peripheral Blood Derived Dendritic Cells

One of the most striking features of the Rat CMV BMDC model is the exquisite sensitivity of these cells to infection with RCMV. Infection rates of BMDC with

RCMV mirrors those for fibroblasts. Flow cytometry analysis revealed that multiplicities of infection with the RCMV-GFP virus (titered on fibroblasts) equal to one PFU per cell, result in greater than 90% of the BMDC scoring positive for GFP (infection). Comparable experiments executed in Human-DC model systems found rates of infection (as measured by viral gene expression or transgenic GFP expression) closer to 10-15% at MOIs equal to 10 (177, 290). Although the use of endothelial cell propagated HCMV (as compared to lab adapted strains and clinical isolates propagated on fibroblasts) has dramatically improved infection rates of DCs, infection of DCs with HCMV still requires a vast excess of virus compared to RCMV infection of BMDC (205). The majority of HCMV-DC infection models characterize either naturally infected circulating DCs isolated from infected HCMV patients, or *in vitro* differentiated DCs derived from peripheral blood monocytes. The first question addressed is whether the origin of the *in vitro* differentiated DC contributed to the increased susceptibility of BMDC to RCMV infection as compared to HCMV infection rates of PBDC.

To examine possible differences in the infection of DCs derived from different progenitor cells, peripheral blood mononuclear cell derived DC and bone marrow derived DC were infected *in vitro* with RCMV-GFP (MOI equal to 1.0) to monitor infection rates by flow cytometry (Figure 25). While greater than 98% of the Bone Marrow DC infected were GFP positive, fewer than 45% of the Peripheral Blood DC scored positive for GFP. Additionally, the mean fluorescent intensity of GFP expression was reduced by more than 80% as compared to that

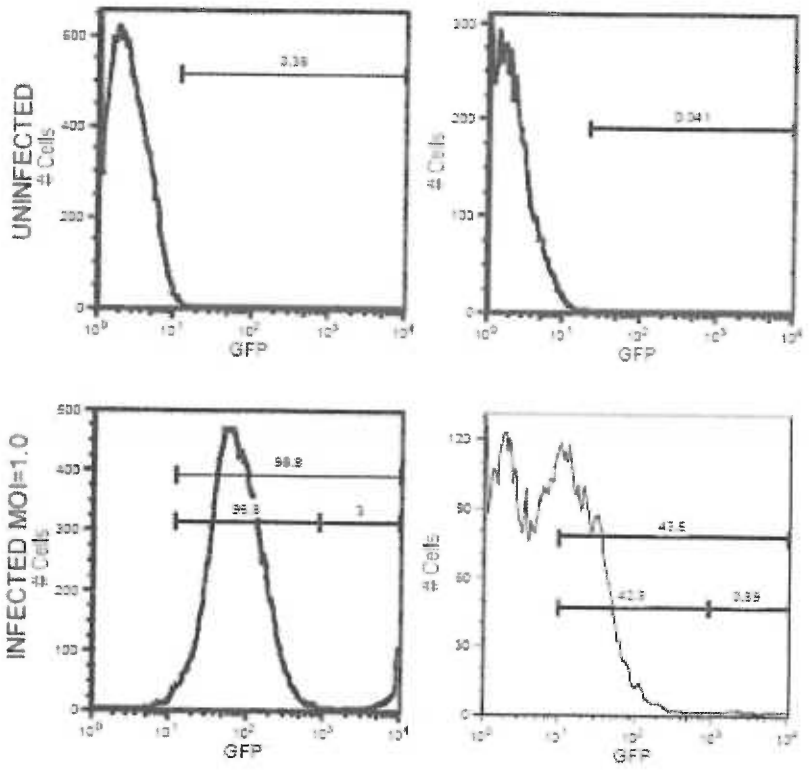


Figure 25. Susceptibility of Bone Marrow Derived Versus Peripheral Blood Derived Dendritic Cell to Rat Cytomegalovirus Infection

Peripheral blood mononuclear cells and bone marrow cells were isolated from the same Lewis strain rat and cultured in parallel in the presence of GM-CSF and IL4. Cells were infected at an MOI equal to 1.0 with RCMV-GFP for 48 hours before flow cytometry analysis of unstained, unpermeabilized cells.

of infected BMDC. The reduced GFP expression observed in PBMC derived DC compared to BMDC could indicate a resistance of the PBDC to viral genome replication, thus reducing the copy number of GFP. Alternatively, the reduction in GFP could be indicative of gene silencing, either of GFP directly or of neighboring ORFs whose transcription requires timed regulation. Quantitative Real Time PCR analysis of cells infected at 12, 24, and 48hpi would yield answers as to the number of viral genomes per cell and the relative rate of increase over time in these two cell types. The GFP results should be confirmed with viral gene expression. Furthermore, a full panel of surface molecule expression to phenotype the differences in PBDC versus BMDC would strengthen the hypothesis that DC derived from different progenitors are phenotypically divergent and as such are not uniformly susceptible to RCMV infection. It would be interesting to determine whether the infection rate of the PBDCs increases by raising the MOI used to infect these cells. This information could be used to determine whether the cultures are uniform in their ability to become infected with RCMV or whether the PBDC are a mixed population of RCMV susceptible and resistant cell types. To truly draw a parallel to HCMV, one would have to perform the complement of experiments in HCMV infected Human BMDC as compared to PBDC. However the finding that Rat CMV infection of PBDC is much less efficient than infection of BMDC, coupled with the reduction in GFP MFI, does suggest a possible explanation for the divergence in infection efficiencies observed between the HCMV (primarily PBDC) and RCMV DC infection models.

B. Down Regulation of MHC II (RT1.B) Surface Expression in Bone Marrow Derived DC Versus Peripheral Blood Derived DC

Another marked contrast between studies investigating HCMV and RCMV down regulation of MHC II in DC is the degree of depletion of cell surface expression. Varani *et al.* have shown that circulating DC isolated from HCMV positive transplant recipients display on average less than 50% of the cell surface MHC II levels as that found on uninfected transplant recipients (289). The degree of down regulation was further confirmed in studies examining PBDC infected *in vitro* with HCMV (184). While on average fewer than 5% of the PBDC were infected *in vitro* at MOIs between 25 and 40, those staining positive for viral antigen (pp52) displayed only a 50% reduction in the amount of surface MHC II compared to their uninfected controls (184). In contrast, infection of BMDC with RCMV results in an average of 90% depletion of cell surface MHC II expression. Several different scenarios could explain cell type differences in MHC II down regulation seen with these various systems. To explore the possibility that differences in progenitor cell type contribute to the variable degree of MHC II surface depletion, RT1.B expression was examined in both BMDC and PBDC infected with RCMV-GFP (Figure 26).

The data presented in Figure 26 demonstrate that while uninfected PBDC express 140% more MHC II (MFI of 579 versus 241) as compared to uninfected

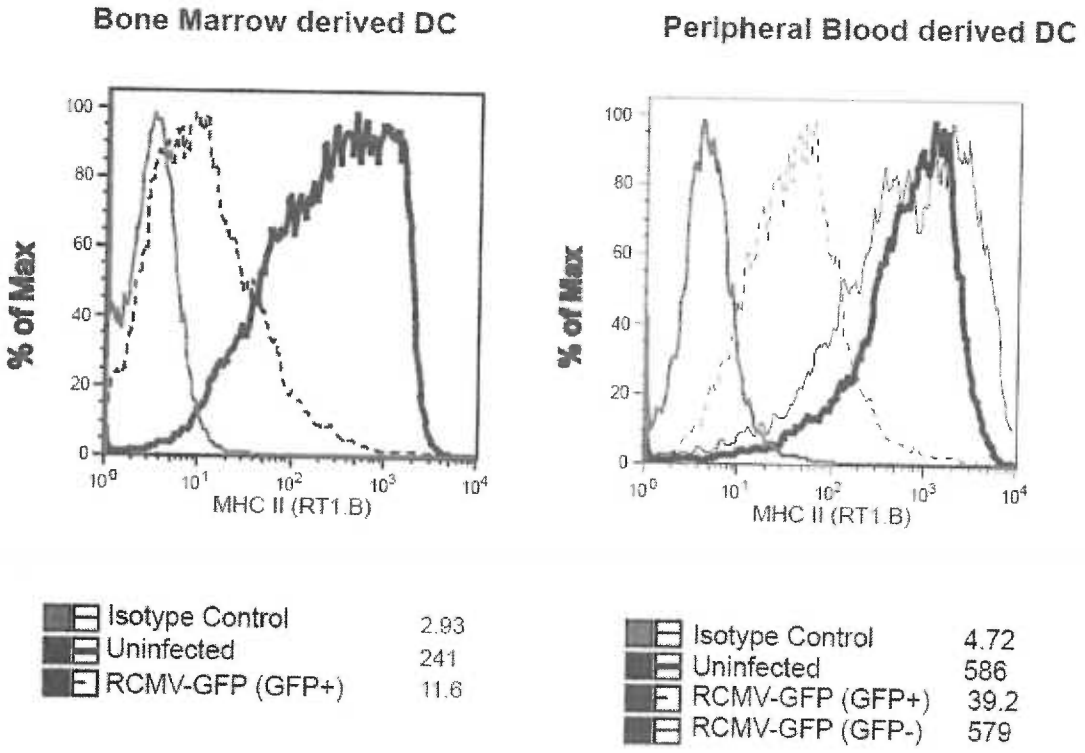


Figure 26. MHC II (RT1.B) Surface Expression in Bone Marrow Derived DC Versus Peripheral Blood Derived DC

Bone marrow cells and peripheral blood mononuclear cells were prepared (as previously described) from the same Lewis strain rat and cultured in parallel in GM-CSF and IL4. Resultant BMDC and PBDC were infected with RCMV-GFP for 48h and then analyzed by flow cytometry for MHC II (RT1.B) surface expression. Geometric mean fluorescent intensities are shown. Due to the high MOI, nearly the entire population of BMDC stained positive for GFP.

MHC II at the surface of uninfected PBDC, the reduction seen at 48hpi is not as complete as in BMDC. However, the greater than one log drop in MHC II surface BMDC on the surface, Rat CMV is able to deplete both PBDC and BMDC MHC II by greater than 93 and 95% respectively. Despite the elevated levels of MHC II in Rat PBDC, the RCMV induced depletion of MHC II in infected (GFP positive) PBDC is still a much more dramatic reduction than has been reported in Human CMV infected PBDC. It would be of great interest to determine if HCMV infection of *bone marrow* derived DC leads to the same degree of MHC II down regulation as seen in *peripheral blood* derived DC, and observed in RCMV. Additionally, further studies need to be completed to determine if RCMV infection of PBDC is saturable, as this appears to be the case for HCMV infected PBDC where increasing the MOI yields only small gains in the percentage of total PBDC infected. If a subpopulation of PBDC is truly resistant to RCMV infection, what is the phenotype of the resistant population? Where is the block in RCMV infection: entry, nuclear translocation, genome replication, virion morphogenesis? Given that the GFP cassette is driven by a cellular promoter, the block in RCMV infection of PBDC is likely an early event in viral infection, pre-morphogenesis.

C. Variable Regulation of HLA-DQ versus HLA-DR Homologues in Rat Cytomegalovirus Infected Bone Marrow derived Dendritic Cells

Although there are five recognized forms of MHC II $\alpha\beta$ heterodimers (HLA-DR, -DQ, -DM, -DP and -DO) only two are commonly found to present antigen at the

surface of APCs: HLA-DQ and HLA-DR (111). Previous studies investigating CMV mediated depletion of cell surface MHC II have all focused on the down regulation of HLA-DR. However, the data presented in the results section of this thesis is centered on the depletion of the rat HLA-DQ homologue, RT1.B. It is possible that the differences witnessed in RCMV down regulation of MHC II versus HCMV induced depletion reflect the variable regulation of the two forms of MHC II. To test this hypothesis, BMDC were infected with the RCMV-GFP virus at low MOI (equal to 0.1) to track the direct effects of infection versus bystander effects due to soluble factors released by the infected cells or through the contact of uninfected cells with infected cells (Figure 27).

Similar to what was observed with RT1.B, Rat CMV depletes RT1.D surface expression to near isotype levels in infected BMDCs (Figure 27). However, unlike data previously described in the results chapter, there appears to be a strong bystander effect on RT1.D expression in uninfected cells in the same culture as infected. This finding mirrors what has been described in the literature for HCMV induced depletion of the human homologue of the rat RT1.D, HLA-DR (38, 206). Further studies will need to be performed to determine if the RCMV RT1.D bystander effect is the result of direct cell-cell contact or that of a soluble factor released by the infected cell. Treatment of uninfected cells with virus free cultured supernatants (taken from infected BMDC conditioned media) combined with neutralizing antibody to the likely culprit, IL10, would help to elucidate if indeed a soluble factor is able to mediate the down-regulation of RT1.D as has

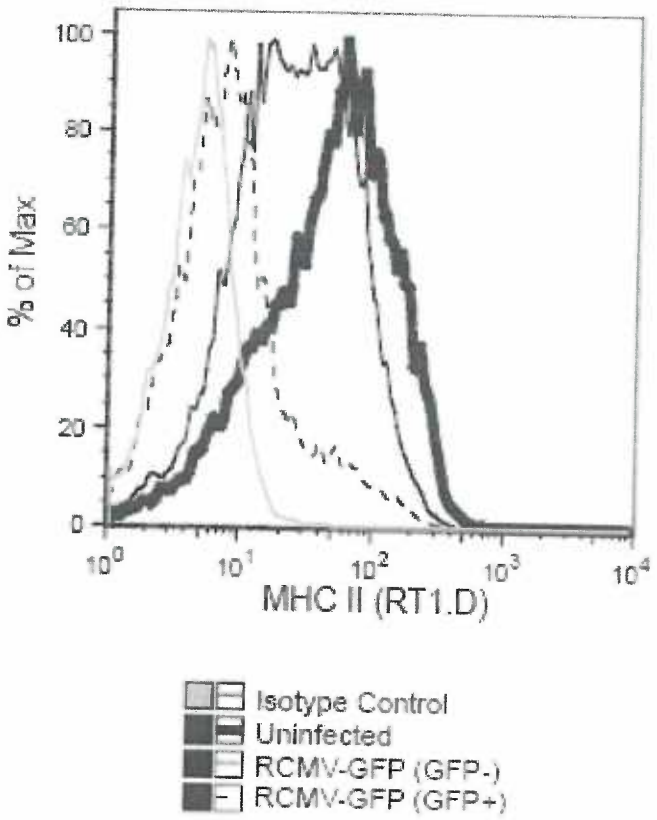


Figure 27. MHC II (RT1.D) Surface Expression in RCMV-GFP Infected Bone Marrow derived Dendritic Cells

Bone Marrow derived Dendritic Cells were either mock treated or infected with RCMV-GFP at an MOI equal to 0.1. Cell surface expression of RT1.D was analyzed by flow cytometry 48hpi. BMDC in the infected culture were gated for GFP expression (GFP positive: infected, GFP minus: uninfected cells in the same culture) and analyzed for RT1.D cell surface expression.

been shown for HLA-DR. Although IL10 is known to down regulate HLA-DR surface expression, not much is known about IL10 regulation of HLA-DQ. Early experiments with recombinant IL10, suggest that RT1.B (HLA-DQ homologue) is not subject to IL10 induced down regulation (data not shown). The observed down regulation of MHC I in response to treatment of BMDC with rat recombinant IL10 (rrIL10) indicates that rrIL0 is functioning at an effective dose (25ng/ml). However the preponderance of literature on IL10 induced down regulation of MHC II (HLA-DR) and the lack of effect on RT1.B precludes a definitive interpretation of the data at this time. An observed effect on RT1.D would suggest differential regulation of the two forms of MHC II in rat DCs.

D. Infection at Low MOI Reveals Both Induction and Down Regulation of Cell Surface CD86 in Infected BMDC Cultures

Studies investigating MCMV infection of the mouse macrophage-monocyte cell line, RAW264.7, identified a spliced gene, m147.5, which mediates the down regulation of the costimulatory molecule, CD86 (151). Derived from two exons in the m149 and m147 ORFs, m147.5 encodes a 145 amino acid protein. Insertion of premature stop codons into the m147.5 coding region of wildtype virus selectively blocked CD86 down regulation. Additionally, ectopic insertion of the m147.5 coding region into the original large scale deletion mutant (affecting ORFs m144-m158) rescued the ability of the deletion mutant to down regulate CD86. The region of h omology within the Rat Cytomegalovirus genome

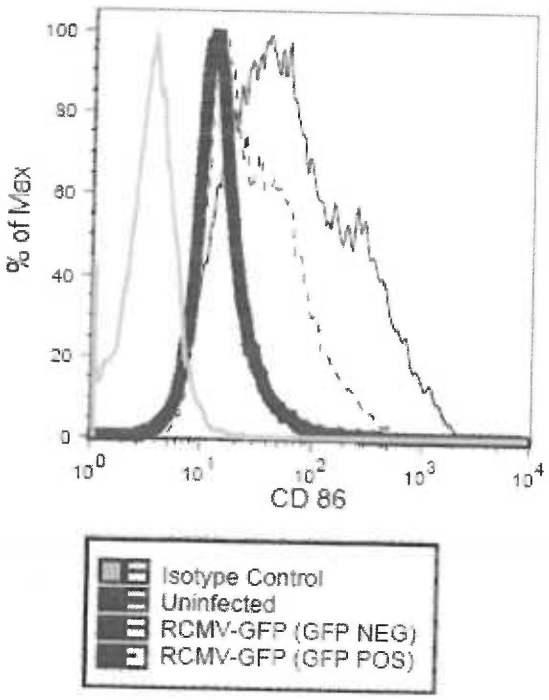


Figure 28. Direct and Indirect Effects of RCMV Infection on CD86 Cell Surface Expression

BMDC were infected with RCMV-GFP at an MOI equal to 0.1 for 48 hours. Both infected and uninfected cultures were analyzed by flow cytometry for surface staining of CD86. RCMV-GFP infected cultures were gated for GFP expression and the GFP positive (GFP POS, thin dashed line) population was compared to both the GFP negative (GFP NEG, thin black line) cells in the same culture and cells from a separate mock infected (Uninfected, thick black line) culture. Isotype control is shown in gray.

corresponding to m147, r147, was truncated in the RCMV-GFP mutant virus. The initial characterization of RCMV infection of BMDC utilized an MOI of 1.0 (versus 0.1) to better compare between the GFP mutant and wildtype virus, which could not be gated for infected versus uninfected cells in the same culture. Early assessment at the higher MOI showed an equivalent increase in CD86 expression in both the RCMV-GFP and wildtype infected cells as compared to uninfected immature BMDCs. Later analysis with the RCMV-GFP virus at lower MOI (equal to 0.1) revealed both an induction and specific depletion of CD86 expression in the infected cultures (Figure 28). While both infected (GFP positive) and uninfected BMDCs (GFP negative) in the same culture expressed elevated levels of CD86 as compared to cells in parallel uninfected cultures, the GFP positive cells expressed 50% less CD86 (as measured by MFI) compared to GFP negative cells in the same culture. Despite the deletion of r147, homologue of the only identified modulator of CD86, RCMV-GFP is still able to counter the induced upregulation of CD86 seen in the infected culture. Large scale deletion analysis of the RCMV genome, coupled with flow cytometry analysis of CD86 expression should uncover additional ORFs whose products mediate CD86 depletion. Additionally, it is not entirely clear if the reduced levels of CD86 in GFP positive cells versus GFP negative cells in the same culture is the result of an active depletion of CD86 or a failure to induce CD86 expression.

E. Infection Blocks LPS Induced Upregulation of BMDC Antigen Presentation

Immature dendritic cells (iDC) reside in the periphery whereupon an encounter with a pathogen triggers iDC migration to the lymphoid organs and concomitant maturation (93). Cell surface expression of costimulatory molecules typically remains low until stimulated to mature through recognition of a pathogen in the periphery, or upon LPS (lipopolysaccharide) stimulation *in vitro*. While dendritic cells express high levels of MHC I and II in both their mature and immature state, maturation signals the release of intracellular stores of MHC to the plasma membrane and stabilizes $\alpha\beta$ heterodimers at the cell surface by down regulating the recycling pathway (288, 308). While earlier data indicates that RCMV effectively down regulates antigen presentation in immature BMDC, the question remained whether or not infection of immature BMDC could block LPS-induced maturation. To answer this question, immature BMDC were infected (or mock infected) for 48 hours before treatment with LPS (at 0.1μg/ml) or mock treated for 24 hours before flow cytometry analysis.

Cell surface expression of MHC I, MHC II (RT1.B) and CD86 was used as a measure of DC maturation (Figure 29). Upon LPS stimulation of uninfected BMDC, MHC I mean fluorescent intensities (MFIs) increased 49.8% (269 in untreated BMDC versus 403 in LPS treated BMDC). MHC II levels increased 12.4% in LPS stimulated BMDC, while the most dramatic increase was observed in CD86 levels where LPS stimulation resulted in a 516% increase in MFI (6.2 in

untreated versus 38.3 in LPS treated BMDC). In contrast to the elevated levels observed in LPS treated uninfected BMDC, infected BMDC were resistant to induction of MHC I and MHC II. Both LPS treated and mock treated infected BMDC maintained the reduced levels of MHC I and II (MHC II MFIs 77% and 87% lower than mock or LPS treated uninfected controls). MHC I and MHC II MFIs in infected BMDC actually dropped 8% upon LPS stimulation. While CD86 surface expression was elevated in infected untreated BMDC (MFI equal to 11.2) compared to uninfected untreated BMDC (MFI equal to 6.21), stimulation with LPS resulted in a 516% increase in uninfected BMDC CD86 MFI, while LPS stimulation of infected BMDC resulted in a 6.3% drop in CD86 MFI. These data indicate that RCMV infection blocks LPS induced upregulation of antigen presentation molecules, MHCI, MHC II and CD86, suggesting that RCMV infection blocks LPS induced DC maturation. Futther analysis of DC function including expression of adhesion molecules important to DC migration, upregulation of other costimulatory molecules (such as CD80) and mixed leukocyte reactions would confirm this preliminary data indicating a block in maturation.

F. Variable Effect of RCMV Infection on Antigen Presentation in LPS Matured Bone Marrow Derived Dendritic Cells

During the initial characterization of the BMDC/RCMV infection model the ability of RCMV to infect both LPS matured and immature BMDC was explored. While

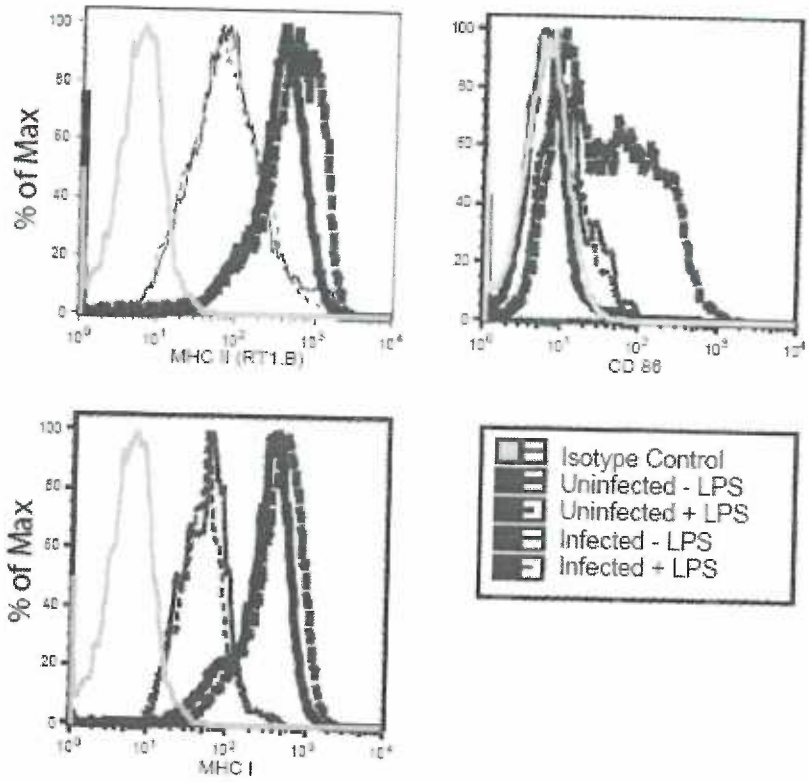


Figure 29. Effect of RCMV Infection on LPS Induced Maturation of BMDC

BMDC were infected with RCMV-GFP or mock infected for 48 hours before treatment with LPS (0.1 μg/ml) or mock. Cells were analyzed by flow cytometry 24 hours post LPS treatment (72 hours post infection). Cell surface staining of MHC II (RT1.B), MHC I and CD86 are shown.

LPS matured BMDC were less susceptible to infection compared to immature BMDC, still a large proportion of the population infected with RCMV-GFP were GFP positive. The study presented in Chapter 2 of this thesis focused on RCMV infection of immature BMDC for two reasons: increased susceptibility to infection coupled with the increased likelihood that DC encounter RCMV in the periphery, where immature DC reside. However, the possibility remains that mature DCs may also encounter RCMV in the lymphoid organs. To address this possibility, the effects of RCMV infection on antigen presentation in LPS matured BMDC were examined (Figure 30).

To ascertain whether RCMV infection of BMDC and subsequent MHC down regulation was dependent on the maturation state of the cell, immature BMDC were treated for 24 hours with LPS (1μg/ml) or mock and then infected with RCMV or mock for 48 hours before flow cytometry analysis of antigen resulted in an 80% drop in MHC II MFI in LPS treated BMDC and a 74% drop in presentation. MHC I data reflected that observed with MHC II, in that LPS treated BMDC infected with RCMV had a 77% lower MFI than uninfected LPS treated BMDC. Immature BMDC infected with RCMV had 69% lower MHC I MFI than uninfected. Interestingly, the LPS stimulated and infected BMDC had uniformly lower MHC I and MHC II MFIs as compared to immature cells. This was quite unexpected, but may reflect the host cell shut down of MHC II recycling. In contrast to the MHC I and II data, CD86 levels were still upregulated in LPS stimulated infected BMDC as compared to

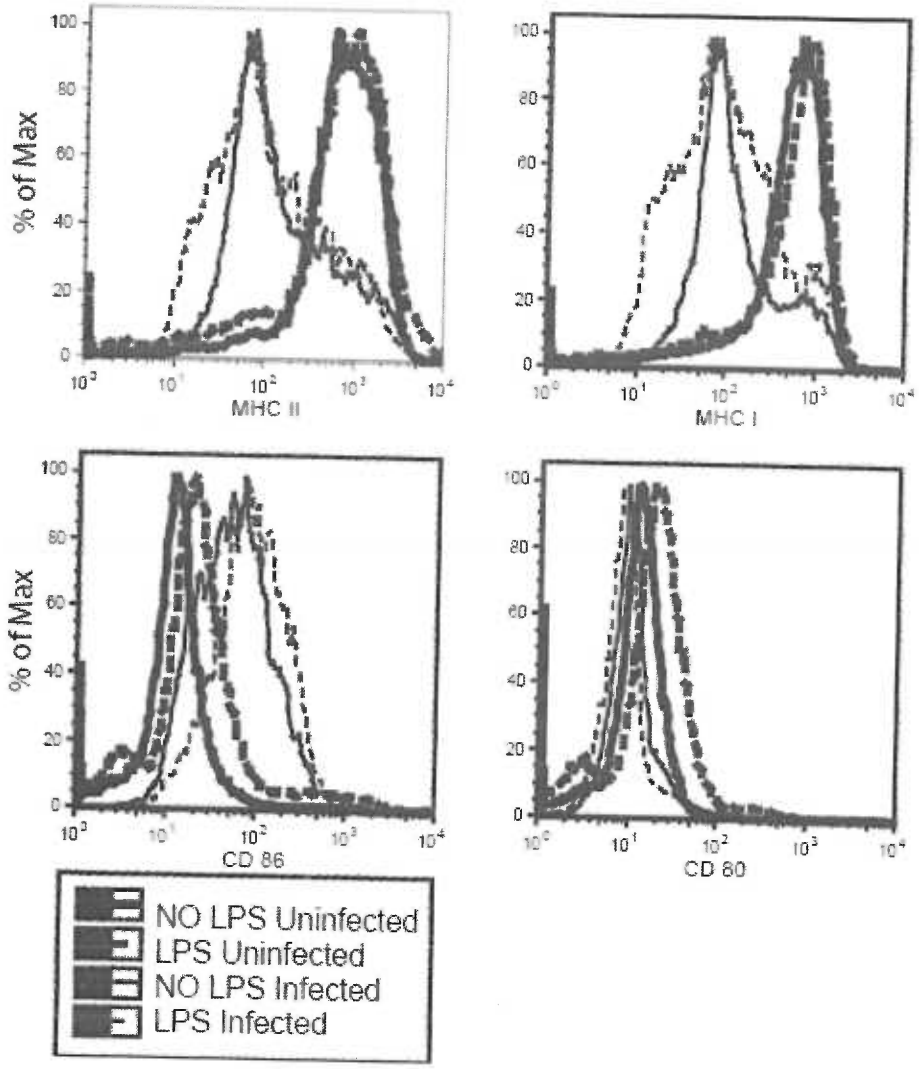


Figure 30. Effects of RCMV Infection on Antigen Presentation in LPS Matured Versus Immature Bone Marrow Derived Dendritic Cells

BMDC were LPS (1.0 μg/ml) or mock treated for 24 hours before infection with RCMV-GFP or mock. Cells were analyzed by flow cytometry 48 hours post infection (72 hours post LPS treatment) for cell surface MHC II (RT1.B), MHC I, CD86 and CD80 infected BMDC.

infected unstimulated BMDC. Together, these results suggest that RCMV infection of both LPS matured and immature BMDC results in the depletion of MHC I and MHC II surface levels equally well, while the effect on antigen presentation does not appear to be a uniform or global down regulation of molecules involved in antigen presentation (as observed in CD86 upregulation in infected BMDC).

REFERENCES

1. **Aaronson, D. S., and C. M. Horvath.** 2002. A road map for those who don't know JAK-STAT. Science **296:**1653-5.

2. **Abate, D. A., S. Watanabe, and E. S. Mocarski.** 2004. Major human cytomegalovirus structural protein pp65 (ppUL83) prevents interferon response factor 3 activation in the interferon response. J Virol **78:**10995-1006.

3. **Abenes, G., M. Lee, E. Haghjoo, T. Tong, X. Zhan, and F. Liu.** 2001. Murine cytomegalovirus open reading frame M27 plays an important role in growth and virulence in mice. J Virol **75:**1697-707.

4. **Adair, R., E. R. Douglas, J. B. Maclean, S. Y. Graham, J. D. Aitken, F. E. Jamieson, and D. J. Dargan.** 2002. The products of human cytomegalovirus genes UL23, UL24, UL43 and US22 are tegument components. J Gen Virol **83:**1315-24.

5. **Adler, S. P.** 1988. Molecular epidemiology of cytomegalovirus: viral transmission among children attending a day care center, their parents, and caretakers. J Pediatr **112:**366-72.

6. **Ahn, K., A. Angulo, P. Ghazal, P. A. Peterson, Y. Yang, and K. Fruh.** 1996. Human cytomegalovirus inhibits antigen presentation by a sequential multistep process. Proc Natl Acad Sci U S A **93:**10990-5.

7. **Ahn, K., A. Angulo, P. Ghazal, P. A. Peterson, Y. Yang, and K. Fruh.** 1996. Human cytomegalovirus inhibits antigen presentation by a sequential multistep process. Proc Natl Acad Sci U S A **93:**10990-5.

8. **Amakawa, R., A. Hakem, T. M. Kundig, T. Matsuyama, J. J. Simard, E. Timms, A. Wakeham, H. W. Mittruecker, H. Griesser, H. Takimoto, R. Schmits, A. Shahinian, P. Ohashi, J. M. Penninger, and T. W. Mak.** 1996. Impaired negative selection of T cells in Hodgkin's disease antigen CD30-deficient mice. Cell **84:**551-62.

9. **Andrews, D. M., C. E. Andoniou, F. Granucci, P. Ricciardi-Castagnoli, and M. A. Degli-Esposti.** 2001. Infection of dendritic cells by murine cytomegalovirus induces functional paralysis. Nat Immunol **2:**1077-84.

10. **Armstrong, D., M. Ely, and L. Steger.** 1971. Post-Transfusion Cytomegaloviremia and Persistence of Cytomegalovirus in Blood. Infect Immun **3:**159-163.

11. **Atalay, R., A. Zimmermann, M. Wagner, E. Borst, C. Benz, M. Messerle, and H. Hengel.** 2002. Identification and expression of human cytomegalovirus transcription units coding for two distinct Fcgamma receptor homologs. J Virol **76:**8596-608.

12. **Baldanti, F., A. Sarasini, E. Silini, M. Barbi, A. Lazzarin, K. K. Biron, and G. Gerna.** 1995. Four dually resistant human cytomegalovirus strains from AIDS patients: single mutations in UL97 and UL54 open reading frames are responsible for ganciclovir- and foscarnet-specific resistance, respectively. Scand J Infect Dis Suppl **99:**103-4.

13. **Bancroft, G. J., G. R. Shellam, and J. E. Chalmer.** 1981. Genetic influences on the augmentation of natural killer (NK) cells during murine cytomegalovirus infection: correlation with patterns of resistance. J Immunol **126:**988-94.

14. **Beck, K., U. Meyer-Konig, M. Weidmann, C. Nern, and F. T. Hufert.** 2003. Human cytomegalovirus impairs dendritic cell function: a novel mechanism of human cytomegalovirus immune escape. Eur J Immunol **33:**1528-38.

15. **Berzofsky, J. A. a. B., Ira J.** 2003. Immunogenicity and Antigen Structure, p. 631-684. *In* W. E. Paul (ed.), Fundamental Immunology, Fifth ed. Lippincott, Williams & Wilkins, Philadelphia.

16. **Biron, C. A., K. S. Byron, and J. L. Sullivan.** 1989. Severe herpesvirus infections in an adolescent without natural killer cells. New Eng J Med **320:**1731-5.

17. **Boehme, K. W., and T. Compton.** 2004. Innate sensing of viruses by toll-like receptors. J Virol **78:**7867-73.

18. **Boland, G. J., R. A. de Weger, M. G. Tilanus, C. Ververs, K. Bosboom-Kalsbeek, and G. C. de Gast.** 1992. Detection of cytomegalovirus (CMV) in granulocytes by polymerase chain reaction compared with the CMV antigen test. J Clin Microbiol **30:**1763-7.

19. **Bolovan-Fritts, C. A., E. S. Mocarski, and J. A. Wiedeman.** 1999. Peripheral blood CD14(+) cells from healthy subjects carry a circular conformation of latent cytomegalovirus genome. Blood **93:**394-8.

20. **Boname, J. M., and P. G. Stevenson.** 2001. MHC class I ubiquitination by a viral PHD/LAP finger protein. Immunity **15:**627-36.

21. **Boppana, S. B., R. F. Pass, W. J. Britt, S. Stagno, and C. A. Alford.** 1992. Symptomatic congenital cytomegalovirus infection: neonatal morbidity and mortality. Pediatr Infect Dis J **11:**93-9.

22. **Boppana, S. B., L. B. Rivera, K. B. Fowler, M. Mach, and W. J. Britt.** 2001. Intrauterine transmission of cytomegalovirus to infants of women with preconceptional immunity. N Engl J Med **344:**1366-71.

23. **Boss, J. M.** 1997. Regulation of transcription of MHC class II genes. Curr Opin Immunol **9:**107-13.

24. **Britt, W.** 2006. Human Cytomegalovirus Infections and Mechanisms of Disease, p. 1-28, Cytomegaloviruses Molecular Biology and Immunology. Caister Academic Press, Norfolk.

25. **Britt, W.** 2006. Primate Models: Pros and Cons, p. 591-606, Cytomegaloviruses Molecular Biology and Immunology. Caister Academic Press, Norfolk.

26. **Britt, W. J., and S. Boppana.** 2004. Human cytomegalovirus virion proteins. Hum Immunol **65:**395-402.

27. **Brown, M. G., A. O. Dokun, J. W. Heusel, H. R. Smith, D. L. Beckman, E. A. Blattenberger, C. E. Dubbelde, L. R. Stone, A. A. Scalzo, and W. M. Yokoyama.** 2001. Vital involvement of a natural killer cell activation receptor in resistance to viral infection. Science **292:**934-7.

28. **Browne, E. P., and T. Shenk.** 2003. Human cytomegalovirus UL83-coded pp65 virion protein inhibits antiviral gene expression in infected cells. Proc Natl Acad Sci U S A **100:**11439-44.

29. **Browne, E. P., B. Wing, D. Coleman, and T. Shenk.** 2001. Altered cellular mRNA levels in human cytomegalovirus-infected fibroblasts: viral block to the accumulation of antiviral mRNAs. J Virol **75:**12319-30.

30. **Brune, W., C. Menard, J. Heesemann, and U. H. Koszinowski.** 2001. A ribonucleotide reductase homolog of cytomegalovirus and endothelial cell tropism. Science **291:**303-5.

31. **Brune, W., C. Menard, J. Heesemann, and U. H. Koszinowski.** 2001. A ribonucleotide reductase homolog of cytomegalovirus and endothelial cell tropism. Science **291:**303-5.

32. **Bruning, J. H., C. A. Bruggeman, C. P. van Boven, and P. J. van Breda Vriesman.** 1986. Passive transfer of cytomegalovirus by cardiac and renal organ transplants in a rat model. Transplantation **41:**695-8.

33. **Bukowski, J. F., B. A. Woda, and R. M. Welsh.** 1984. Pathogenesis of murine cytomegalovirus infection in natural killer cell-depleted mice. Journal of Virology **52:**119-28.

34. **Cai, W. H., B. Gu, and S. Person.** 1988. Role of glycoprotein B of herpes simplex virus type 1 in viral entry and cell fusion. J Virol **62:**2596-604.

35. **Cavanaugh, V. J., R. M. Stenberg, T. L. Staley, H. W. t. Virgin, M. R. MacDonald, S. Paetzold, H. E. Farrell, W. D. Rawlinson, and A. E. Campbell.** 1996. Murine cytomegalovirus with a deletion of genes

spanning HindIII-J and - I displays altered cell and tissue tropism. J Virol **70:**1365-74.

36. **Cebulla, C. M., D. M. Miller, Y. Zhang, B. M. Rahill, P. Zimmerman, J. M. Robinson, and D. D. Sedmak.** 2002. Human cytomegalovirus disrupts constitutive MHC class II expression. J Immunol **169:**167-76.

37. **Chambers, J., A. Angulo, D. Amaratunga, H. Guo, Y. Jiang, J. S. Wan, A. Bittner, K. Frueh, M. R. Jackson, P. A. Peterson, M. G. Erlander, and P. Ghazal.** 1999. DNA microarrays of the complex human cytomegalovirus genome: profiling kinetic class with drug sensitivity of viral gene expression. J Virol **73:**5757-66.

38. **Chang, W. L., N. Baumgarth, D. Yu, and P. A. Barry.** 2004. Human cytomegalovirus-encoded interleukin-10 homolog inhibits maturation of dendritic cells and alters their functionality. J Virol **78:**8720-31.

39. **Chapuis, F., M. Rosenzwajg, M. Yagello, M. Ekman, P. Biberfeld, and J. C. Gluckman.** 1997. Differentiation of human dendritic cells from monocytes in vitro. Eur J Immunol **27:**431-41.

40. **Child, S. J., M. Hakki, K. L. De Niro, and A. P. Geballe.** 2004. Evasion of cellular antiviral responses by human cytomegalovirus TRS1 and IRS1. J Virol **78:**197-205.

41. **Coligan, J. E., T. J. Kindt, H. Uehara, J. Martinko, and S. G. Nathenson.** 1981. Primary structure of a murine transplantation antigen. Nature **291:**35-9.

42. **Compton, T., R. R. Nepomuceno, and D. M. Nowlin.** 1992. Human cytomegalovirus penetrates host cells by pH-independent fusion at the cell surface. Virology **191:**387-95.

43. **Coscoy, L., D. J. Sanchez, and D. Ganem.** 2001. A novel class of herpesvirus-encoded membrane-bound E3 ubiquitin ligases regulates endocytosis of proteins involved in immune recognition. J Cell Biol **155:**1265-73.

44. **Cresswell, P.** 1994. Assembly, transport, and function of MHC class II molecules. Annu Rev Immunol **12:**259-93.

45. **Dabbagh, K., M. E. Dahl, P. Stepick-Biek, and D. B. Lewis.** 2002. Toll-like receptor 4 is required for optimal development of Th2 immune responses: role of dendritic cells. J Immunol **168:**4524-30.

46. **Dalod, M., T. Hamilton, R. Salomon, T. P. Salazar-Mather, S. C. Henry, J. D. Hamilton, and C. A. Biron.** 2003. Dendritic cell responses to early murine cytomegalovirus infection: subset functional specialization and differential regulation by interferon alpha/beta. J Exp Med **197:**885-98.

47. **Dalod, M., T. P. Salazar-Mather, L. Malmgaard, C. Lewis, C. Asselin-Paturel, F. Briere, G. Trinchieri, and C. A. Biron.** 2002. Interferon alpha/beta and interleukin 12 responses to viral infections: pathways regulating dendritic cell cytokine expression in vivo. J Exp Med **195:**517-28.

48. **Daniels, K. A., G. Devora, W. C. Lai, C. L. O'Donnell, M. Bennett, and R. M. Welsh.** 2001. Murine cytomegalovirus is regulated by a discrete

subset of natural killer cells reactive with monoclonal antibody to Ly49H. Journal of Experimental Medicine **194:**29-44.

49. **Davison, A. J., A. Dolan, P. Akter, C. Addison, D. J. Dargan, D. J. Alcendor, D. J. McGeoch, and G. S. Hayward.** 2003. The human cytomegalovirus genome revisited: comparison with the chimpanzee cytomegalovirus genome. J Gen Virol **84:**17-28.

50. **De Gassart, A., V. Camosseto, J. Thibodeau, M. Ceppi, N. Catalan, P. Pierre, and E. Gatti.** 2008. MHC class II stabilization at the surface of human dendritic cells is the result of maturation-dependent MARCH I down-regulation. Proc Natl Acad Sci U S A **105:**3491-6.

51. **del Val, M., H. Hengel, H. Hacker, U. Hartlaub, T. Ruppert, P. Lucin, and U. H. Koszinowski.** 1992. Cytomegalovirus prevents antigen presentation by blocking the transport of peptide-loaded major histocompatibility complex class I molecules into the medial-Golgi compartment. J Exp Med **176:**729-38.

52. **Dolan, A., C. Cunningham, R. D. Hector, A. F. Hassan-Walker, L. Lee, C. Addison, D. J. Dargan, D. J. McGeoch, D. Gatherer, V. C. Emery, P. D. Griffiths, C. Sinzger, B. P. McSharry, G. W. Wilkinson, and A. J. Davison.** 2004. Genetic content of wild-type human cytomegalovirus. J Gen Virol **85:**1301-12.

53. **Dubois, B., B. Vanbervliet, J. Fayette, C. Massacrier, C. Van Kooten, F. Briere, J. Banchereau, and C. Caux.** 1997. Dendritic cells enhance

growth and differentiation of CD40-activated B lymphocytes. J Exp Med **185:**941-51.

54. **Dunn, C., N. J. Chalupny, C. L. Sutherland, S. Dosch, P. V. Sivakumar, D. C. Johnson, and D. Cosman.** 2003. Human cytomegalovirus glycoprotein UL16 causes intracellular sequestration of NKG2D ligands, protecting against natural killer cell cytotoxicity. J Exp Med **197:**1427-39.

55. **Dunn, W., C. Chou, H. Li, R. Hai, D. Patterson, V. Stolc, H. Zhu, and F. Liu.** 2003. Functional profiling of a human cytomegalovirus genome. Proc Natl Acad Sci U S A **100:**14223-8.

56. **Ejrnaes, M., C. M. Filippi, M. M. Martinic, E. M. Ling, L. M. Togher, S. Crotty, and M. G. von Herrath.** 2006. Resolution of a chronic viral infection after interleukin-10 receptor blockade. J Exp Med **203:**2461-72.

57. **Emery, V. C.** 1999. Viral dynamics during active cytomegalovirus infection and pathology. Intervirology **42:**405-11.

58. **Emery, V. C., C. A. Sabin, A. V. Cope, D. Gor, A. F. Hassan-Walker, and P. D. Griffiths.** 2000. Application of viral-load kinetics to identify patients who develop cytomegalovirus disease after transplantation. Lancet **355:**2032-6.

59. **Erice, A.** 1999. Resistance of human cytomegalovirus to antiviral drugs. Clin Microbiol Rev **12:**286-97.

60. **Facchetti, F., C. de Wolf-Peeters, D. Y. Mason, K. Pulford, J. J. van den Oord, and V. J. Desmet.** 1988. Plasmacytoid T cells.

Immunohistochemical evidence for their monocyte/macrophage origin. Am J Pathol **133:**15-21.

61. **Farrell, H. E., H. Vally, D. M. Lynch, P. Fleming, G. R. Shellam, A. A. Scalzo, and N. J. Davis-Poynter.** 1997. Inhibition of natural killer cells by a cytomegalovirus MHC class I homologue in vivo. Nature **386:**510-4.

62. **Favoreel, H. W., G. R. Van de Walle, H. J. Nauwynck, and M. B. Pensaert.** 2003. Virus complement evasion strategies. J Gen Virol **84:**1-15.

63. **Fayette, J., B. Dubois, S. Vandenabeele, J. M. Bridon, B. Vanbervliet, I. Durand, J. Banchereau, C. Caux, and F. Briere.** 1997. Human dendritic cells skew isotype switching of CD40-activated naive B cells towards IgA1 and IgA2. J Exp Med **185:**1909-18.

64. **Feire, A. L., H. Koss, and T. Compton.** 2004. Cellular integrins function as entry receptors for human cytomegalovirus via a highly conserved disintegrin-like domain. Proc Natl Acad Sci U S A **101:**15470-5.

65. **Fennie, E. H., Y. S. Lie, M. A. Low, P. Gribling, and K. P. Anderson.** 1988. Reduced mortality in murine cytomegalovirus infected mice following prophylactic murine interferon-gamma treatment. Antiviral Res **10:**27-39.

66. **Fernandez, N. C., A. Lozier, C. Flament, P. Ricciardi-Castagnoli, D. Bellet, M. Suter, M. Perricaudet, T. Tursz, E. Maraskovsky, and L. Zitvogel.** 1999. Dendritic cells directly trigger NK cell functions: cross-talk

relevant in innate anti-tumor immune responses in vivo. Nat Med **5:**405-11.

67. **Filippi, C. M., and M. G. von Herrath.** 2008. IL-10 and the resolution of infections. J Pathol **214:**224-30.

68. **Fish, K. N., W. Britt, and J. A. Nelson.** 1996. A novel mechanism for persistence of human cytomegalovirus in macrophages. J Virol **70:**1855-62.

69. **Fish, K. N., C. Soderberg-Naucler, L. K. Mills, S. Stenglein, and J. A. Nelson.** 1998. Human cytomegalovirus persistently infects aortic endothelial cells. J Virol **72:**5661-8.

70. **Flint, S., Enquist, LW, Racaniello, VR, and Skalka, AM.** Principles of Virology, Second ed. ASM Press, Washington, D.C.

71. **Fontes, J. D., S. Kanazawa, N. Nekrep, and B. M. Peterlin.** 1999. The class II transactivator CIITA is a transcriptional integrator. Microbes Infect **1:**863-9.

72. **Forrester, A., H. Farrell, G. Wilkinson, J. Kaye, N. Davis-Poynter, and T. Minson.** 1992. Construction and properties of a mutant of herpes simplex virus type 1 with glycoprotein H coding sequences deleted. J Virol **66:**341-8.

73. **Frascaroli, G., S. Varani, A. Mastroianni, S. Britton, D. Gibellini, G. Rossini, M. P. Landini, and C. Soderberg-Naucler.** 2006. Dendritic cell function in cytomegalovirus-infected patients with mononucleosis. J Leukoc Biol **79:**932-40.

74. **Fruh, K., E. Bartee, K. Gouveia, and M. Mansouri.** 2002. Immune evasion by a novel family of viral PHD/LAP-finger proteins of gamma-2 herpesviruses and poxviruses. Virus Res **88:**55-69.

75. **Gamadia, L. E., E. B. Remmerswaal, J. F. Weel, F. Bemelman, R. A. van Lier, and I. J. Ten Berge.** 2003. Primary immune responses to human CMV: a critical role for IFN-gamma-producing CD4+ T cells in protection against CMV disease. Blood **101:**2686-92.

76. **Geginat, G., T. Ruppert, H. Hengel, R. Holtappels, and U. H. Koszinowski.** 1997. IFN-gamma is a prerequisite for optimal antigen processing of viral peptides in vivo. J Immunol **158:**3303-10.

77. **Gibson, W.** 2006. Assembly and Maturation of the Capsid, p. 231-244. *In* M. Reddehase (ed.), Cytomegaloviruses Molecular Biology and Immunology. Caister Academic Press, Norfolk.

78. **Goodrum, F., C. T. Jordan, S. S. Terhune, K. High, and T. Shenk.** 2004. Differential outcomes of human cytomegalovirus infection in primitive hematopoietic cell subpopulations. Blood **104:**687-95.

79. **Goodrum, F. D., C. T. Jordan, K. High, and T. Shenk.** 2002. Human cytomegalovirus gene expression during infection of primary hematopoietic progenitor cells: A model for latency. Proc Natl Acad Sci U S A **99:**16255-16260.

80. **Granucci, F., C. Vizzardelli, N. Pavelka, S. Feau, M. Persico, E. Virzi, M. Rescigno, G. Moro, and P. Ricciardi-Castagnoli.** 2001. Inducible IL-

2 production by dendritic cells revealed by global gene expression analysis. Nat Immunol **2:**882-8.

81. **Grefte, A., M. van der Giessen, W. van Son, and T. H. The.** 1993. Circulating cytomegalovirus (CMV)-infected endothelial cells in patients with an active CMV infection. J Infect Dis **167:**270-7.

82. **Griffith, B. P., H. L. Lucia, and G. D. Hsiung.** 1982. Brain and visceral involvement during congenital cytomegalovirus infection of guinea pigs. Pediatr Res **16:**455-9.

83. **Griffiths, P. D., and C. Baboonian.** 1984. A prospective study of primary cytomegalovirus infection during pregnancy: final report. Br J Obstet Gynaecol **91:**307-15.

84. **Gruhler, A., P. A. Peterson, and K. Fruh.** 2000. Human cytomegalovirus immediate early glycoprotein US3 retains MHC class I molecules by transient association. Traffic **1:**318-25.

85. **Gyorkey, F., J. L. Melnick, G. A. Guinn, P. Gyorkey, and M. E. DeBakey.** 1984. Herpesviridae in the endothelial and smooth muscle cells of the proximal aorta in arteriosclerotic patients. Exp. Mol. Pathol. **40:**328-339.

86. **Hahn, G., H. Khan, F. Baldanti, U. H. Koszinowski, M. G. Revello, and G. Gerna.** 2002. The human cytomegalovirus ribonucleotide reductase homolog UL45 is dispensable for growth in endothelial cells, as determined by a BAC-cloned clinical isolate of human cytomegalovirus with preserved wild-type characteristics. J Virol **76:**9551-5.

87. **Hahn, G., M. G. Revello, M. Patrone, E. Percivalle, G. Campanini, A. Sarasini, M. Wagner, A. Gallina, G. Milanesi, U. Koszinowski, F. Baldanti, and G. Gerna.** 2004. Human cytomegalovirus UL131-128 genes are indispensable for virus growth in endothelial cells and virus transfer to leukocytes. J Virol **78:**10023-33.

88. **Hakki, M., and A. P. Geballe.** 2005. Double-stranded RNA binding by human cytomegalovirus pTRS1. J Virol **79:**7311-8.

89. **Hakki, M., E. E. Marshall, K. L. De Niro, and A. P. Geballe.** 2006. Binding and nuclear relocalization of protein kinase R by human cytomegalovirus TRS1. J Virol **80:**11817-26.

90. **Hamano, S., H. Yoshida, H. Takimoto, K. Sonoda, K. Osada, X. He, Y. Minamishima, G. Kimura, and K. Nomoto.** 1998. Role of macrophages in acute murine cytomegalovirus infection. Microbiol Immunol **42:**607-16.

91. **Hamilton, J. D., and B. J. Seaworth.** 1985. Transmission of latent cytomegalovirus in a murine kidney tissue transplantation model. Transplantation **39:**290-6.

92. **Hansen, S. G., L. I. Strelow, D. C. Franchi, D. G. Anders, and S. W. Wong.** 2003. Complete sequence and genomic analysis of rhesus cytomegalovirus. J Virol **77:**6620-36.

93. **Hanson, L. K., B. L. Dalton, Z. Karabekian, H. E. Farrell, W. D. Rawlinson, R. M. Stenberg, and A. E. Campbell.** 1999. Transcriptional analysis of the murine cytomegalovirus HindIII-I region: identification of a novel immediate-early gene region. Virology **260:**156-64.

94. **Hanson, L. K., J. S. Slater, Z. Karabekian, G. Ciocco-Schmitt, and A. E. Campbell.** 2001. Products of US22 genes M140 and M141 confer efficient replication of murine cytomegalovirus in macrophages and spleen. J Virol **75:**6292-302.

95. **Hanson, L. K., J. S. Slater, Z. Karabekian, H. W. t. Virgin, C. A. Biron, M. C. Ruzek, N. van Rooijen, R. P. Ciavarra, R. M. Stenberg, and A. E. Campbell.** 1999. Replication of murine cytomegalovirus in differentiated macrophages as a determinant of viral pathogenesis. J Virol **73:**5970-80.

96. **Hanson, L. K., J. S. Slater, Z. Karabekian, H. W. t. Virgin, C. A. Biron, M. C. Ruzek, N. van Rooijen, R. P. Ciavarra, R. M. Stenberg, and A. E. Campbell.** 1999. Replication of murine cytomegalovirus in differentiated macrophages as a determinant of viral pathogenesis. J Virol **73:**5970-80.

97. **Hassink, G. C., J. G. Duijvestijn-van Dam, D. Koppers-Lalic, J. van Gaans-van den Brink, D. van Leeuwen, C. Vink, C. A. Bruggeman, and E. J. Wiertz.** 2005. Rat cytomegalovirus induces a temporal downregulation of major histocompatibility complex class I cell surface expression. Viral Immunol **18:**607-15.

98. **Hegde, N. R., C. Dunn, D. M. Lewinsohn, M. A. Jarvis, J. A. Nelson, and D. C. Johnson.** 2005. Endogenous human cytomegalovirus gB is presented efficiently by MHC class II molecules to CD4+ CTL. J Exp Med **202:**1109-19.

99. **Hegde, N. R., and D. C. Johnson.** 2003. Human cytomegalovirus US2 causes similar effects on both major histocompatibility complex class I and II proteins in epithelial and glial cells. J Virol **77:**9287-94.

100. **Hegde, N. R., R. A. Tomazin, T. W. Wisner, C. Dunn, J. M. Boname, D. M. Lewinsohn, and D. C. Johnson.** 2002. Inhibition of HLA-DR assembly, transport, and loading by human cytomegalovirus glycoprotein US3: a novel mechanism for evading major histocompatibility complex class II antigen presentation. J Virol **76:**10929-41.

101. **Heise, M. T., J. L. Pollock, A. O'Guin, M. L. Barkon, S. Bormley, and H. W. t. Virgin.** 1998. Murine cytomegalovirus infection inhibits IFN gamma-induced MHC class II expression on macrophages: the role of type I interferon. Virology **241:**331-44.

102. **Hendrix, M., P. H. J. Dormans, P. Kitseelar, F. Bosman, and C. A. Bruggeman.** 1989. The presence of CMV nucleic acids arterial walls of atherosclerotic and non-atherosclerotic patients. Am. J. Path. **134:**1151-7.

103. **Hengel, H., C. Esslinger, J. Pool, E. Goulmy, and U. H. Koszinowski.** 1995. Cytokines restore MHC class I complex formation and control antigen presentation in human cytomegalovirus-infected cells. J Gen Virol **76:**2987-97.

104. **Hengel, H., P. Lucin, S. Jonjic, T. Ruppert, and U. H. Koszinowski.** 1994. Restoration of cytomegalovirus antigen presentation by gamma interferon combats viral escape. J Virol **68:**289-97.

105. **Hilkens, C. M., P. Kalinski, M. de Boer, and M. L. Kapsenberg.** 1997. Human dendritic cells require exogenous interleukin-12-inducing factors to direct the development of naive T-helper cells toward the Th1 phenotype. Blood **90:**1920-6.

106. **Hirsch, M. S., R. T. Schooley, A. B. Cosimi, P. S. Russell, F. L. Delmonico, N. E. Tolkoff-Rubin, J. T. Herrin, K. Cantell, M. L. Farrell, T. R. Rota, and R. H. Rubin.** 1983. Effects of interferon-alpha on cytomegalovirus reactivation syndromes in renal-transplant recipients. N Engl J Med **308:**1489-93.

107. **Hodson, E. M., C. A. Jones, A. C. Webster, G. F. Strippoli, P. G. Barclay, K. Kable, D. Vimalachandra, and J. C. Craig.** 2005. Antiviral medications to prevent cytomegalovirus disease and early death in recipients of solid-organ transplants: a systematic review of randomised controlled trials. Lancet **365:**2105-15.

108. **Hruban, R. H., T. C. Wu, W. E. Beschorner, D. E. Cameron, R. F. Ambinder, W. A. Baumgartner, B. A. Reitz, and G. M. Hutchins.** 1990. Cytomegalovirus nucleic acids in allografted hearts. Hum Pathol **21:**981-2.

109. **Isaacs, A., and J. Lindenmann.** 1957. Virus interference. I. The interferon. Proc R Soc Lond B Biol Sci **147:**258-67.

110. **Jabrane-Ferrat, N., N. Nekrep, G. Tosi, L. J. Esserman, and B. M. Peterlin.** 2002. Major histocompatibility complex class II transcriptional platform: assembly of nuclear factor Y and regulatory factor X (RFX) on DNA requires RFX5 dimers. Mol Cell Biol **22:**5616-25.

111. **Janeway, C. A., P. Travers, M. Walport, and M. Shlomchik.** 2001. Immunobiology: the immune system in health and disease, 5th ed. Garland Publishing, New York.

112. **Jarvis, M. A., and J. A. Nelson.** 2002. Human cytomegalovirus persistence and latency in endothelial cells and macrophages. Curr Opin Microbiol **5:**403-7.

113. **Jarvis, M. A., and J. A. Nelson.** 2002. Mechanisms of human cytomegalovirus persistence and latency. Front Biosci **7:**d1575-82.

114. **Jesoniek, and Kiolemenoglou.** 1904. Über einen Befund von protozoenartigen Gebilden in der organen eines heriditär-luetischen Fötus. Münschener Medizinische Wochenschrift.**:**1905-7.

115. **Johnson, D. C., and N. R. Hegde.** 2002. Inhibition of the MHC class II antigen presentation pathway by human cytomegalovirus. Curr Top Microbiol Immunol **269:**101-15.

116. **Johnson, D. C., and A. B. Hill.** 1998. Herpesvirus evasion of the immune system. Curr Top Microbiol Immunol **232:**149-77.

117. **Johnson, D. C., and M. W. Ligas.** 1988. Herpes simplex viruses lacking glycoprotein D are unable to inhibit virus penetration: quantitative evidence for virus-specific cell surface receptors. J Virol **62:**4605-12.

118. **Jones, T. R., E. J. Wiertz, L. Sun, K. N. Fish, J. A. Nelson, and H. L. Ploegh.** 1996. Human cytomegalovirus US3 impairs transport and maturation of major histocompatibility complex class I heavy chains. PNAS USA **93:**11327-33.

119. **Jonjic, S., I. Pavic, B. Polic, I. Crnkovic, P. Lucin, and U. H. Koszinowski.** 1994. Antibodies are not essential for the resolution of primary cytomegalovirus infection but limit dissemination of recurrent virus. J Exp Med **179:**1713-7.

120. **Jordan, M. C., W. Rousseau, J. A. Stewart, G. R. Noble, and T. D. Chin.** 1973. Spontaneous cytomegalovirus mononucleosis. Clinical and laboratory observations in nine cases. Ann Intern Med **79:**153-60.

121. **Kaptein, S. J. F., Vink, Cornelius, Bruggeman, Cathrien A., and Stassen, Frank R.M.** 2006. The Rat Model for CMV Infection and Vascular Disease, p. 501-524, Cytomegaloviruses Molecular Biology and Immunology. Caister Academic Press, Norfolk.

122. **Kasinrerk, W., T. Baumruker, O. Majdic, W. Knapp, and H. Stockinger.** 1993. CD1 molecule expression on human monocytes induced by granulocyte-macrophage colony-stimulating factor. J Immunol **150:**579-84.

123. **Kavanagh, D. G., M. C. Gold, M. Wagner, U. H. Koszinowski, and A. B. Hill.** 2001. The multiple immune-evasion genes of murine cytomegalovirus are not redundant: m4 and m152 inhibit antigen presentation in a complementary and cooperative fashion. J Exp Med **194:**967-78.

124. **Kavanagh, D. G., U. H. Koszinowski, and A. B. Hill.** 2001. The murine cytomegalovirus immune evasion protein m4/gp34 forms biochemically distinct complexes with class I MHC at the cell surface and in a pre-Golgi compartment. J Immunol **167:**3894-902.

125. **Kisseleva, T., S. Bhattacharya, J. Braunstein, and C. W. Schindler.** 2002. Signaling through the JAK/STAT pathway, recent advances and future challenges. Gene **285:**1-24.

126. **Kleijnen, M. F., J. B. Huppa, P. Lucin, S. Mukherjee, H. Farrell, A. E. Campbell, U. H. Koszinowski, A. B. Hill, and H. L. Ploegh.** 1997. A mouse cytomegalovirus glycoprotein, gp34, forms a complex with folded class I MHC molecules in the ER which is not retained but is transported to the cell surface. Embo J **16:**685-94.

127. **Klemola, E., R. Von Essen, G. Henle, and W. Henle.** 1970. Infectious-mononucleosis-like disease with negative heterophil agglutination test. Clinical features in relation to Epstein-Barr virus and cytomegalovirus antibodies. J Infect Dis **121:**608-14.

128. **Klenovsek, K., F. Weisel, A. Schneider, U. Appelt, S. Jonjic, M. Messerle, B. Bradel-Tretheway, T. H. Winkler, and M. Mach.** 2007. Protection from CMV infection in immunodeficient hosts by adoptive transfer of memory B cells. Blood **110:**3472-9.

129. **Kloover, J. S., J. L. Hillebrands, G. de Wit, G. Grauls, J. Rozing, C. A. Bruggeman, and P. Nieuwenhuis.** 2000. Rat cytomegalovirus replication in the salivary glands is exclusively confined to striated duct cells [In Process Citation]. Virchows Arch **437:**413-21.

130. **Kondo, K., H. Kaneshima, and E. S. Mocarski.** 1994. Human cytomegalovirus latent infection of granulocyte-macrophage progenitors. Proc Natl Acad Sci U S A **91:**11879-83.

131. **Koszinowski, U. H., M. Del Val, and M. J. Reddehase.** 1990. Cellular and molecular basis of the protective immune response to cytomegalovirus infection. Current Topics in Microbiology & Immunology **154:**189-220.

132. **Kotenko, S. V., S. Saccani, L. S. Izotova, O. V. Mirochnitchenko, and S. Pestka.** 2000. Human cytomegalovirus harbors its own unique IL-10 homolog (cmvIL-10). Proc Natl Acad Sci U S A **97:**1695-700.

133. **Krmpotic, A., D. H. Busch, I. Bubic, F. Gebhardt, H. Hengel, M. Hasan, A. A. Scalzo, U. H. Koszinowski, and S. Jonjic.** 2002. MCMV glycoprotein gp40 confers virus resistance to CD8+ T cells and NK cells in vivo. Nat Immunol **3:**529-35.

134. **Krmpotic, A., M. Hasan, A. Loewendorf, T. Saulig, A. Halenius, T. Lenac, B. Polic, I. Bubic, A. Kriegeskorte, E. Pernjak-Pugel, M. Messerle, H. Hengel, D. H. Busch, U. H. Koszinowski, and S. Jonjic.** 2005. NK cell activation through the NKG2D ligand MULT-1 is selectively prevented by the glycoprotein encoded by mouse cytomegalovirus gene m145. J Exp Med **201:**211-20.

135. **Kropshofer, H., S. O. Arndt, G. Moldenhauer, G. J. Hammerling, and A. B. Vogt.** 1997. HLA-DM acts as a molecular chaperone and rescues empty HLA-DR molecules at lysosomal pH. Immunity **6:**293-302.

136. **Lang, D. J., P. A. Ebert, B. M. Rodgers, H. P. Boggess, and R. S. Rixse.** 1977. Reduction of postperfusion cytomegalovirus-infections following the use of leukocyte depleted blood. Transfusion **17:**391-5.

137. **Le Roy, E., A. Muhlethaler-Mottet, C. Davrinche, B. Mach, and J. L. Davignon.** 1999. Escape of human cytomegalovirus from HLA-DR-restricted CD4(+) T-cell response is mediated by repression of gamma interferon-induced class II transactivator expression. J Virol **73:**6582-9.

138. **Lee, S., J. Yoon, B. Park, Y. Jun, M. Jin, H. C. Sung, I. H. Kim, S. Kang, E. J. Choi, B. Y. Ahn, and K. Ahn.** 2000. Structural and functional dissection of human cytomegalovirus US3 in binding major histocompatibility complex class I molecules. J Virol **74:**11262-9.

139. **Lee, S. H., S. Girard, D. Macina, M. Busa, A. Zafer, A. Belouchi, P. Gros, and S. M. Vidal.** 2001. Susceptibility to mouse cytomegalovirus is associated with deletion of an activating natural killer cell receptor of the C-type lectin superfamily. Nat Genet **28:**42-5.

140. **Lembo, D., M. Donalisio, A. Hofer, M. Cornaglia, W. Brune, U. Koszinowski, L. Thelander, and S. Landolfo.** 2004. The ribonucleotide reductase R1 homolog of murine cytomegalovirus is not a functional enzyme subunit but is required for pathogenesis. J Virol **78:**4278-88.

141. **Lewandoski, M.** 2001. Conditional control of gene expression in the mouse. Nat Rev Genet **2:**743-55.

142. **Ligas, M. W., and D. C. Johnson.** 1988. A herpes simplex virus mutant in which glycoprotein D sequences are replaced by beta-galactosidase sequences binds to but is unable to penetrate into cells. J Virol **62:**1486-94.

143. **Lilley, B. N., and H. L. Ploegh.** 2005. Viral modulation of antigen presentation: manipulation of cellular targets in the ER and beyond. Immunol Rev **207:**126-44.

144. **Lilley, B. N., H. L. Ploegh, and R. S. Tirabassi.** 2001. Human Cytomegalovirus Open Reading Frame TRL11/IRL11 Encodes an Immunoglobulin G Fc-Binding Protein. J Virol **75:**11218-21.

145. **Liu, T., K. M. Khanna, X. Chen, D. J. Fink, and R. L. Hendricks.** 2000. CD8(+) T cells can block herpes simplex virus type 1 (HSV-1) reactivation from latency in sensory neurons. J Exp Med **191:**1459-66.

146. **Lockridge, K. M., G. Sequar, S. S. Zhou, Y. Yue, C. P. Mandell, and P. A. Barry.** 1999. Pathogenesis of experimental rhesus cytomegalovirus infection. J Virol **73:**9576-83.

147. **Lockridge, K. M., S. S. Zhou, R. H. Kravitz, J. L. Johnson, E. T. Sawai, E. L. Blewett, and P. A. Barry.** 2000. Primate cytomegaloviruses encode and express an IL-10-like protein. Virology **268:**272-80.

148. **Lodoen, M., K. Ogasawara, J. A. Hamerman, H. Arase, J. P. Houchins, E. S. Mocarski, and L. L. Lanier.** 2003. NKG2D-mediated natural killer cell protection against cytomegalovirus is impaired by viral gp40 modulation of retinoic acid early inducible 1 gene molecules. J Exp Med **197:**1245-53.

149. **Lodoen, M. B., G. Abenes, S. Umamoto, J. P. Houchins, F. Liu, and L. L. Lanier.** 2004. The cytomegalovirus m155 gene product subverts

natural killer cell antiviral protection by disruption of H60-NKG2D interactions. J Exp Med **200:**1075-81.

150. **Lodoen, M. B., and L. L. Lanier.** 2005. Viral modulation of NK cell immunity. Nat Rev Microbiol **3:**59-69.

151. **Loewendorf, A., C. Kruger, E. M. Borst, M. Wagner, U. Just, and M. Messerle.** 2004. Identification of a mouse cytomegalovirus gene selectively targeting CD86 expression on antigen-presenting cells. J Virol **78:**13062-71.

152. **Lucin, P., S. Jonjic, M. Messerle, B. Polic, H. Hengel, and U. H. Koszinowski.** 1994. Late phase inhibition of murine cytomegalovirus replication by synergistic action of interferon-gamma and tumour necrosis factor. J Gen Virol **75:**101-10.

153. **Lucin, P., I. Pavic, B. Polic, S. Jonjic, and U. H. Koszinowski.** 1992. Gamma interferon-dependent clearance of cytomegalovirus infection in salivary glands. J Virol **66:**1977-84.

154. **Lugo-Villarino, G., R. Maldonado-Lopez, R. Possemato, C. Penaranda, and L. H. Glimcher.** 2003. T-bet is required for optimal production of IFN-gamma and antigen-specific T cell activation by dendritic cells. Proc Natl Acad Sci U S A **100:**7749-54.

155. **Margulies, D. H., and McCluskey, James.** 2003. The Major Histocompatibility Complex and its Encoded Proteins, p. 571-612. *In* W. E. Paul (ed.), Fundamental Immunology, Fifth ed. Lippincott, Williams & Wilkins, Philadelphia.

156. **Mathys, S., T. Schroeder, J. Ellwart, U. H. Koszinowski, M. Messerle, and U. Just.** 2003. Dendritic cells under influence of mouse cytomegalovirus have a physiologic dual role: to initiate and to restrict T cell activation. J Infect Dis **187:**988-99.

157. **Matsuki, Y., M. Ohmura-Hoshino, E. Goto, M. Aoki, M. Mito-Yoshida, M. Uematsu, T. Hasegawa, H. Koseki, O. Ohara, M. Nakayama, K. Toyooka, K. Matsuoka, H. Hotta, A. Yamamoto, and S. Ishido.** 2007. Novel regulation of MHC class II function in B cells. Embo J **26:**846-54.

158. **McLaughlin, K., C. Wu, G. Fick, N. Muirhead, D. Hollomby, and A. Jevnikar.** 2002. Cytomegalovirus seromismatching increases the risk of acute renal allograft rejection. Transplantation **74:**813-6.

159. **McRae, B. L., R. T. Semnani, M. P. Hayes, and G. A. van Seventer.** 1998. Type I IFNs inhibit human dendritic cell IL-12 production and Th1 cell development. J Immunol **160:**4298-304.

160. **Melnick, J. L., E. Adam, and M. E. Debakey.** 1993. Cytomegalovirus and atherosclerosis. Eur Heart J **14 Suppl K:**30-8.

161. **Menard, C., M. Wagner, Z. Ruzsics, K. Holak, W. Brune, A. E. Campbell, and U. H. Koszinowski.** 2003. Role of Murine Cytomegalovirus US22 Gene Family Members in Replication in Macrophages. J. Virol. **77:**5557-5570.

162. **Menard, C., M. Wagner, Z. Ruzsics, K. Holak, W. Brune, A. E. Campbell, and U. H. Koszinowski.** 2003. Role of murine

cytomegalovirus US22 gene family members in replication in macrophages. J Virol **77:**5557-70.

163. **Merigan, T. C., D. G. Renlund, S. Keay, and e. al.** 1992. A controlled trial of ganciclovir to prevent cytomegalovirus disease after heart transplantation. N Engl J Med **326:**1182-6.

164. **Mettenleiter, T. C., B. G. Klupp, and H. Granzow.** 2006. Herpesvirus assembly: a tale of two membranes. Curr Opin Microbiol **9:**423-9.

165. **Michel, D. a. M., Thomas.** 2006. Antiviral Intervention, Resistance, and Perspectives, p. 573-590, Cytomegaloviruses Molecular Biology and Immunology. Caister Academic Press, Norfolk.

166. **Michelson, S.** 1997. Interaction of human cytomegalovirus with monocytes/macrophages: a love-hate relationship. Pathol Biol (Paris) **45:**146-58.

167. **Miller, D. M., B. M. Rahill, J. M. Boss, M. D. Lairmore, J. E. Durbin, J. W. Waldman, and D. D. Sedmak.** 1998. Human cytomegalovirus inhibits major histocompatibility complex class II expression by disruption of the Jak/Stat pathway. J Exp Med **187:**675-83.

168. **Mitchison, N. A., and J. Roes.** 2002. Patterned variation in murine MHC promoters. Proc Natl Acad Sci U S A **99:**10561-6.

169. **Mocarski, E. S.** 2001. Cytomegaloviruses and their replication., p. 2629-2673. *In* B. N. Fields (ed.), Virology, 4th ed, vol. 2. Lippincott-Raven, Philadelphia.

170. **Mocarski, E. S., Jr.** 2004. Immune escape and exploitation strategies of cytomegaloviruses: impact on and imitation of the major histocompatibility system. Cell Microbiol **6:**707-17.

171. **Mocarski, E. S., Jr.** 2002. Immunomodulation by cytomegaloviruses: manipulative strategies beyond evasion. Trends Microbiol **10:**332-9.

172. **Mocarski, J., Edward S., Hahn, Gabriele, Lofgren White, Kirsten, Xu, Jiake, Slobedman, Barry, Hertel, Laura, Aguirre, Shirley A. and Noda, Satoshi.** 2006. Myeloid Cell Recruitment and Function in Pathogenesis, p. 465-482, Cytomegaloviruses Molecular Biology and Immunology. Caister Academic Press.

173. **Mocarski Jr., E. S.** 2006. Comparative analysis of herpesvirus-common proteins, p. 44-58. *In* A. Arvin, Mocarski, ES, Moore, P. et al. (ed.), Human Herpesviruses: Biology, Therapy and Immunoprophylaxis. Cambridge Press, Cambridge.

174. **Mocarski Jr., E. S.** 2006. Viral genes and their functions, p. 202-228. *In* A. Arvin, Mocarski, ES, Moore, P. et al. (ed.), Human Herpesviruses: Biology, Therapy and Immunoprophylaxis. Cambridge Press, Cambridge.

175. **Mocarski Jr., E. S., Shenk, Thomas, and Pass, Robert F.** 2007. Cytomegaloviruses, p. 2701-2772. *In* D. M. Knipe, and Howley, Peter M. (ed.), Fields Virology, Fifth ed, vol. Two. Lippincott, Williams & Wilkins, Philadelphia.

176. **Moutaftsi, M., P. Brennan, S. A. Spector, and Z. Tabi.** 2004. Impaired lymphoid chemokine-mediated migration due to a block on the chemokine

receptor switch in human cytomegalovirus-infected dendritic cells. J Virol **78:**3046-54.

177. **Moutaftsi, M., A. M. Mehl, L. K. Borysiewicz, and Z. Tabi.** 2002. Human cytomegalovirus inhibits maturation and impairs function of monocyte-derived dendritic cells. Blood **99:**2913-21.

178. **Mulamba, G. B., A. Hu, R. F. Azad, K. P. Anderson, and D. M. Coen.** 1998. Human cytomegalovirus mutant with sequence-dependent resistance to the phosphorothioate oligonucleotide fomivirsen (ISIS 2922). Antimicrob Agents Chemother **42:**971-3.

179. **Muthana, M., B. Fairburn, S. Mirza, L. K. Slack, and A. G. Pockley.** 2004. Systematic evaluation of the conditions required for the generation of immature rat bone marrow-derived dendritic cells and their phenotypic and functional characterization. J Immunol Methods **294:**165-79.

180. **Myerson, D., R. C. Hackman, J. A. Nelson, D. C. Ward, and J. K. McDougall.** 1984. Widespread presence of histologically occult cytomegalovirus. Hum Pathol **15:**430-9.

181. **Nagano, Y., and Y. Kojima.** 1958. [Inhibition of vaccinia infection by a liquid factor in tissues infected by homologous virus.]. C R Seances Soc Biol Fil **152:**1627-9.

182. **Nordoy, I., H. Rollag, E. Lien, H. Sindre, M. Degre, P. Aukrust, S. S. Froland, and F. Muller.** 2003. Cytomegalovirus infection induces production of human interleukin-10 in macrophages. Eur J Clin Microbiol Infect Dis **22:**737-41.

183. **Ochando, J. C., C. Homma, Y. Yang, A. Hidalgo, A. Garin, F. Tacke, V. Angeli, Y. Li, P. Boros, Y. Ding, R. Jessberger, G. Trinchieri, S. A. Lira, G. J. Randolph, and J. S. Bromberg.** 2006. Alloantigen-presenting plasmacytoid dendritic cells mediate tolerance to vascularized grafts. Nat Immunol **7**:652-62.

184. **Odeberg, J., B. Plachter, L. Branden, and C. Soderberg-Naucler.** 2003. Human cytomegalovirus protein pp65 mediates accumulation of HLA-DR in lysosomes and destruction of the HLA-DR alpha-chain. Blood **101**:4870-7.

185. **Odeberg, J., and C. Soderberg-Naucler.** 2001. Reduced expression of HLA class II molecules and linterleukin-10- and transforming growth factor beta1-independent suppression of T-cell proliferation in human cytomegalovirus-infected macrophage cultures. J Virol **75**:5174-81.

186. **Ogawa-Goto, K., K. Tanaka, W. Gibson, E. Moriishi, Y. Miura, T. Kurata, S. Irie, and T. Sata.** 2003. Microtubule network facilitates nuclear targeting of human cytomegalovirus capsid. J Virol **77**:8541-7.

187. **Olding, L. B., F. C. Jensen, and M. B. Oldstone.** 1975. Pathogenesis of of cytomegalovirus infection. I. Activation of virus from bone marrow-derived lymphocytes by in vitro allogenic reaction. J Exp Med **141**:561-72.

188. **Onno, M., C. Pangault, G. Le Friec, V. Guilloux, P. Andre, and R. Fauchet.** 2000. Modulation of HLA-G antigens expression by human cytomegalovirus: specific induction in activated macrophages harboring human cytomegalovirus infection. J Immunol **164**:6426-34.

189. **Orloff, S. L., D. N. Streblow, C. Soderberg-Naucler, Q. Yin, C. Kreklywich, C. L. Corless, P. A. Smith, C. B. Loomis, L. K. Mills, J. W. Cook, C. A. Bruggeman, J. A. Nelson, and C. R. Wagner.** 2002. Elimination of donor-specific alloreactivity prevents cytomegalovirus-accelerated chronic rejection in rat small bowel and heart transplants. Transplantation **73:**679-88.

190. **Pande, N. T., C. Powers, K. Ahn, and K. Fruh.** 2005. Rhesus cytomegalovirus contains functional homologues of US2, US3, US6, and US11. J Virol **79:**5786-98.

191. **Pannuti, C. S., L. S. Vilas Boas, M. J. Angelo, V. Amato Neto, G. C. Levi, J. S. de Mendonca, and C. V. de Godoy.** 1985. Cytomegalovirus mononucleosis in children and adults: differences in clinical presentation. Scand J Infect Dis **17:**153-6.

192. **Park, B., S. Lee, E. Kim, K. Cho, S. R. Riddell, S. Cho, and K. Ahn.** 2006. Redox regulation facilitates optimal peptide selection by MHC class I during antigen processing. Cell **127:**369-82.

193. **Paul, W. E.** 2003. The Immune System: An Introduction, p. 1-22. *In* W. E. Paul (ed.), Fundamental Immunology, Fifth ed. Lippincott Williams & Wilkins, Philadelphia.

194. **Paul, W. E.** 2003. The Immune System: An Introduction. *In* W. E. Paul (ed.), Fundamental Immunology, 5th ed. Lippincott, Williams, and Wilkins, Philadelphia.

195. **Paulus, C., S. Krauss, and M. Nevels.** 2006. A human cytomegalovirus antagonist of type I IFN-dependent signal transducer and activator of transcription signaling. Proc Natl Acad Sci U S A **103:**3840-5.

196. **Peckham, C. S., K. S. Chin, J. C. Coleman, K. Henderson, R. Hurley, and P. M. Preece.** 1983. Cytomegalovirus infection in pregnancy: preliminary findings from a prospective study. Lancet **1:**1352-5.

197. **Pinto, A. K., and A. B. Hill.** 2005. Viral interference with antigen presentation to CD8+ T cells: lessons from cytomegalovirus. Viral Immunol **18:**434-44.

198. **Pinto, A. K., M. W. Munks, U. H. Koszinowski, and A. B. Hill.** 2006. Coordinated function of murine cytomegalovirus genes completely inhibits CTL lysis. J Immunol **177:**3225-34.

199. **Ploegh, H. L.** 1998. Viral strategies of immune evasion. Science **280:**248-53.

200. **Podlech, J., R. Holtappels, M. F. Pahl-Seibert, H. P. Steffens, and M. J. Reddehase.** 2000. Murine model of interstitial cytomegalovirus pneumonia in syngeneic bone marrow transplantation: persistence of protective pulmonary CD8-T-cell infiltrates after clearance of acute infection. Journal of Virology **74:**7496-507.

201. **Polic, B., H. Hengel, A. Krmpotic, J. Trgovcich, I. Pavic, P. Luccaronin, S. Jonjic, and U. H. Koszinowski.** 1998. Hierarchical and redundant lymphocyte subset control precludes cytomegalovirus replication during latent infection. J Exp Med **188:**1047-54.

202. **Pollock, J. L., R. M. Presti, S. Paetzold, and H. W. t. Virgin.** 1997. Latent murine cytomegalovirus infection in macrophages. Virology **227:**168-79.

203. **Powers, C. a. F., K.** 2008. Signal Peptide-Dependent Inhibition of MHC Class I Heavy Chain Translation by Rhesus Cytomegalovirus. PLoS Pathogens **4:**e1000150.

204. **Presti, R. M., J. L. Pollock, A. J. Dal Canto, A. K. O'Guin, and H. W. t. Virgin.** 1998. Interferon gamma regulates acute and latent murine cytomegalovirus infection and chronic disease of the great vessels. J Exp Med **188:**577-88.

205. **Raftery, M. J., M. Schwab, S. M. Eibert, Y. Samstag, H. Walczak, and G. Schonrich.** 2001. Targeting the function of mature dendritic cells by human cytomegalovirus: a multilayered viral defense strategy. Immunity **15:**997-1009.

206. **Raftery, M. J., D. Wieland, S. Gronewald, A. A. Kraus, T. Giese, and G. Schonrich.** 2004. Shaping phenotype, function, and survival of dendritic cells by cytomegalovirus-encoded IL-10. J Immunol **173:**3383-91.

207. **Rapp, F., Messerle, M., Lucin, P., and Koszinowski, U.H.** 1993. In vivo protection studies with MCMV glycoproteins gB and gH expressed by vaccinia virus., p. 327-332. *In* S. a. P. Michelson, S.A. (ed.), Multidisciplinary Approach to Understanding Cytomegalovirus Disease. Excerpta Medica, Amsterdam.

208. **Raulet, D. H.** 2003. Natural Killer Cells. *In* W. E. Paul (ed.), Fundamental Immunology, Fifth ed. Lippincott Williams & Wilkins, Philadelphia.

209. **Reddehase, M.** 2006. From protozoan to proteomics, p. xxiii-xxvii. *In* M. Reddehase (ed.), Cytomegaloviruses Molecular Biology and Immunology. Caister Academic Press, Norfolk.

210. **Reddehase, M. J.** 2000. The immunogenicity of human and murine cytomegaloviruses. Curr Opin Immunol **12:**390-6.

211. **Reddehase, M. J., M. Balthesen, M. Rapp, S. Jonjic, I. Pavic, and U. H. Koszinowski.** 1994. The conditions of primary infection define the load of latent viral genome in organs and the risk of recurrent cytomegalovirus disease. Journal of Experimental Medicine **179:**185-93.

212. **Reddehase, M. J., W. Mutter, K. Munch, H. J. Buhring, and U. H. Koszinowski.** 1987. CD8-positive T lymphocytes specific for murine cytomegalovirus immediate-early antigens mediate protective immunity. J Virol **61:**3102-8.

213. **Redpath, S., A. Angulo, N. R. Gascoigne, and P. Ghazal.** 1999. Murine cytomegalovirus infection down-regulates MHC class II expression on macrophages by induction of IL-10. J Immunol **162:**6701-7.

214. **Rehm, A., A. Engelsberg, D. Tortorella, I. J. Korner, I. Lehmann, H. L. Ploegh, and U. E. Hopken.** 2002. Human cytomegalovirus gene products US2 and US11 differ in their ability to attack major histocompatibility class I heavy chains in dendritic cells. J Virol **76:**5043-50.

215. **Reusch, U., W. Muranyi, P. Lucin, H. G. Burgert, H. Hengel, and U. H. Koszinowski.** 1999. A cytomegalovirus glycoprotein re-routes MHC class I complexes to lysosomes for degradation. Embo J **18:**1081-91.

216. **Reyburn, H. T., O. Mandelboim, M. Vales-Gomez, D. M. Davis, L. Pazmany, and J. L. Strominger.** 1997. The class I MHC homologue of human cytomegalovirus inhibits attack by natural killer cells [see comments]. Nature **386:**514-7.

217. **Ribbert, H.** 1904. Über protizoanartige Zellen in der Niere eines syphilitischen Neugeboren und in der Parotis von Kinden. Zentralbl. Allg. Path. path. Anat. **15:**945-48.

218. **Rice, G. P., R. D. Schrier, and M. B. Oldstone.** 1984. Cytomegalovirus infects human lymphocytes and monocytes: virus expression is restricted to immediate-early gene products. Proc Natl Acad Sci U S A **81:**6134-8.

219. **Roizman, B., L. E. Carmichael, F. Deinhardt, G. de-The, A. J. Nahmias, W. Plowright, F. Rapp, P. Sheldrick, M. Takahashi, and K. Wolf.** 1981. Herpesviridae. Definition, provisional nomenclature, and taxonomy. The Herpesvirus Study Group, the International Committee on Taxonomy of Viruses. Intervirology **16:**201-17.

220. **Rolle, A., M. Mousavi-Jazi, M. Eriksson, J. Odeberg, C. Soderberg-Naucler, D. Cosman, K. Karre, and C. Cerboni.** 2003. Effects of human cytomegalovirus infection on ligands for the activating NKG2D receptor of NK cells: up-regulation of UL16-binding protein (ULBP)1 and ULBP2 is counteracted by the viral UL16 protein. J Immunol **171:**902-8.

221. **Romanowski, M. J., E. Garrido-Guerrero, and T. Shenk.** 1997. pIRS1 and pTRS1 are present in human cytomegalovirus virions. J Virol **71:**5703-5.

222. **Romanowski, M. J., and T. Shenk.** 1997. Characterization of the human cytomegalovirus irs1 and trs1 genes: a second immediate-early transcription unit within irs1 whose product antagonizes transcriptional activation. J Virol **71:**1485-96.

223. **Roop, C., L. Hutchinson, and D. C. Johnson.** 1993. A mutant herpes simplex virus type 1 unable to express glycoprotein L cannot enter cells, and its particles lack glycoprotein H. J Virol **67:**2285-97.

224. **Rott, D., J. Zhu, M. S. Burnett, Y. F. Zhou, A. Zalles-Ganley, J. Ogunmakinwa, and S. E. Epstein.** 2003. Effects of MF-tricyclic, a selective cyclooxygenase-2 inhibitor, on atherosclerosis progression and susceptibility to cytomegalovirus replication in apolipoprotein-E knockout mice. J Am Coll Cardiol **41:**1812-9.

225. **Rowe, W. P., J. W. Hartley, S. Waterman, H. C. Turner, and J. Huebner.** 1956. Cytopathogenic agent resembling human salivary gland virus recovered from tissue cultures of human adenoids. Proc. Soc. Exp. Biol. Med. **92:**418-24.

226. **Ryckman, B. J., M. A. Jarvis, D. D. Drummond, J. A. Nelson, and D. C. Johnson.** 2006. Human cytomegalovirus entry into epithelial and endothelial cells depends on genes UL128 to UL150 and occurs by endocytosis and low-pH fusion. J Virol **80:**710-22.

227. **Ryckman, B. J., B. L. Rainish, M. C. Chase, J. A. Borton, J. A. Nelson, M. A. Jarvis, and D. C. Johnson.** 2008. Characterization of the human cytomegalovirus gH/gL/UL128-131 complex that mediates entry into epithelial and endothelial cells. J Virol **82:**60-70.

228. **Sacher, T., J. Podlech, C. A. Mohr, S. Jordan, Z. Ruzsics, M. J. Reddehase, and U. H. Koszinowski.** 2008. The major virus-producing cell type during murine cytomegalovirus infection, the hepatocyte, is not the source of virus dissemination in the host. Cell Host Microbe **3:**263-72.

229. **Schleiss, M. R., N. Bourne, N. J. Jensen, F. Bravo, and D. I. Bernstein.** 2000. Immunogenicity evaluation of DNA vaccines that target guinea pig cytomegalovirus proteins glycoprotein B and UL83. Viral Immunol **13:**155-67.

230. **Schleiss, M. R., and N. J. Jensen.** 2003. Cloning and expression of the guinea pig cytomegalovirus glycoprotein B (gB) in a recombinant baculovirus: utility for vaccine studies for the prevention of experimental infection. J Virol Methods **108:**59-65.

231. **Schleiss, M. R. a. L., Juan C.** 2006. The Guinea-pig Model of Congenital Cytomegalovirus Infection, p. 525-550, Cytomegaloviruses Molecular Biology and Immunology. Caister Academic Press, Norfolk.

232. **Schmader, K. E., R. Rahija, K. R. Porter, G. Daley, and J. D. Hamilton.** 1992. Aging and reactivation of latent murine cytomegalovirus. J Infect Dis **166:**1403-7.

233. **Schrier, R. D., J. A. Nelson, and M. B. Oldstone.** 1985. Detection of human cytomegalovirus in peripheral blood lymphocytes in a natural infection. Science **230:**1048-51.

234. **Sedmak, D. D., A. M. Guglielmo, D. A. Knight, D. J. Birmingham, E. H. Huang, and W. J. Waldman.** 1994. Cytomegalovirus inhibits major histocompatibility class II expression on infected endothelial cells [see comments]. Am J Pathol **144:**683-92.

235. **Shanley, J. D., M. C. Jordan, and J. G. Stevens.** 1981. Modification by adoptive humoral immunity of murine cytomegalovirus infection. J Infect Dis **143:**231-7.

236. **Shellam, G. R., J. E. Allan, J. M. Papadimitriou, and G. J. Bancroft.** 1981. Increased susceptibility to cytomegalovirus infection in beige mutant mice. Proc Natl Acad Sci U S A **78:**5104-8.

237. **Shin, J. S., M. Ebersold, M. Pypaert, L. Delamarre, A. Hartley, and I. Mellman.** 2006. Surface expression of MHC class II in dendritic cells is controlled by regulated ubiquitination. Nature **444:**115-8.

238. **Shukla, D., and P. G. Spear.** 2001. Herpesviruses and heparan sulfate: an intimate relationship in aid of viral entry. J Clin Invest **108:**503-10.

239. **Siegal, F. P., N. Kadowaki, M. Shodell, P. A. Fitzgerald-Bocarsly, K. Shah, S. Ho, S. Antonenko, and Y. J. Liu.** 1999. The nature of the principal type 1 interferon-producing cells in human blood. Science **284:**1835-7.

240. **Simmons, P., K. Kaushansky, and B. Torok-Storb.** 1990. Mechanisms of cytomegalovirus-mediated myelosuppression: perturbation of stromal cell function versus direct infection of myeloid cells. Proc Natl Acad Sci U S A **87:**1386-90.

241. **Sinzger, C., Digel, M., Jahn, G.** 2008. Cytomegalovirus Cell Tropism, p. 63-83. *In* T. Shenk, Stinski, M.F. (ed.), Human Cytomegalovirus. Springer, Berlin.

242. **Sinzger, C., A. Grefte, B. Plachter, A. S. Gouw, T. H. The, and G. Jahn.** 1995. Fibroblasts, epithelial cells, endothelial cells and smooth muscle cells are major targets of human cytomegalovirus infection in lung and gastrointestinal tissues. J Gen Virol **76:**741-50.

243. **Skaletskaya, A., L. M. Bartle, T. Chittenden, A. L. McCormick, E. S. Mocarski, and V. S. Goldmacher.** 2001. A cytomegalovirus-encoded inhibitor of apoptosis that suppresses caspase-8 activation. Proc Natl Acad Sci U S A **98:**7829-34.

244. **Smith, M. G.** 1954. Propagation of salivary gland virus of the mouse in tissue cultures. Proc Soc Exp Biol Med **86:**435-40.

245. **Smith, M. G.** 1956. Propagation of salivary gland virus of the mouse in tissue cultures. Proc. Soc. Exp. Biol. Med. **86:**435-40.

246. **Smith, P. D., S. S. Saini, M. Raffeld, J. F. Manischewitz, and S. M. Wahl.** 1992. Cytomegalovirus induction of tumor necrosis factor-alpha by human monocytes and mucosal macrophages. J Clin Invest **90:**1642-8.

247. **Söderberg-Naucler, C., K. N. Fish, and J. A. Nelson.** 1997. Reactivation of latent human cytomegalovirus by allogeneic stimulation of blood cells from healthy donors. Cell **91:**119-26.

248. **Söderberg-Naucler, C., D. N. Streblow, K. N. Fish, J. Allan-Yorke, P. P. Smith, and J. A. Nelson.** 2001. Reactivation of latent human cytomegalovirus in CD14(+) monocytes is differentiation dependent. J Virol **75:**7543-54.

249. **Söderberg, C., S. Larsson, S. Bergstedt-Lindqvist, and E. Moller.** 1993. Definition of a subset of human peripheral blood mononuclear cells that are permissive to human cytomegalovirus infection. J Virol **67:**3166-75.

250. **Span, A. H., P. M. Frederik, G. Grauls, G. P. Van Boven, and C. A. Bruggeman.** 1993. CMV induced vascular injury: an electron-microscopic study in the rat. In Vivo **7:**567-73.

251. **Span, A. H., G. Grauls, F. Bosman, C. P. van Boven, and C. A. Bruggeman.** 1992. Cytomegalovirus infection induces vascular injury in the rat. Atherosclerosis **93:**41-52.

252. **Span, A. H., M. C. van Dam-Mieras, W. Mullers, J. Endert, A. D. Muller, and C. A. Bruggeman.** 1991. The effect of virus infection on the adherence of leukocytes or platelets to endothelial cells. Eur J Clin Invest **21:**331-8.

253. **Spear, G. T., N. S. Lurain, C. J. Parker, M. Ghassemi, G. H. Payne, and M. Saifuddin.** 1995. Host cell-derived complement control proteins CD55

and CD59 are incorporated into the virions of two unrelated enveloped viruses. Human T cell leukemia/lymphoma virus type I (HTLV-I) and human cytomegalovirus (HCMV). J Immunol **155:**4376-81.

254. **Spear, P. G., and R. Longnecker.** 2003. Herpesvirus entry: an update. J Virol **77:**10179-85.

255. **Spee, P., J. Subjeck, and J. Neefjes.** 1999. Identification of novel peptide binding proteins in the endoplasmic reticulum: ERp72, calnexin, and grp170. Biochemistry **38:**10559-66.

256. **Spencer, J. V., K. M. Lockridge, P. A. Barry, G. Lin, M. Tsang, M. E. Penfold, and T. J. Schall.** 2002. Potent immunosuppressive activities of cytomegalovirus-encoded interleukin-10. J Virol **76:**1285-92.

257. **Spencer, J. V., K. M. Lockridge, P. A. Barry, G. Lin, M. Tsang, M. E. Penfold, and T. J. Schall.** 2002. Potent immunosuppressive activities of cytomegalovirus-encoded interleukin-10. Journal of Virology **76:**1285-92.

258. **Spiller, O. B., S. M. Hanna, D. V. Devine, and F. Tufaro.** 1997. Neutralization of cytomegalovirus virions: the role of complement. J Infect Dis **176:**339-47.

259. **Spiller, O. B., B. P. Morgan, F. Tufaro, and D. V. Devine.** 1996. Altered expression of host encoded complement regulators on human cytomegalovirus infected cells. Eur J Immunol **26:**1532-8.

260. **Stagno, S., R. F. Pass, G. Cloud, W. J. Britt, R. E. Henderson, P. D. Walton, D. A. Veren, F. Page, and C. A. Alford.** 1986. Primary

cytomegalovirus infection in pregnancy. Incidence, transmission to fetus, and clinical outcome. Jama **256:**1904-8.

261. **Steinmetz, M., J. G. Frelinger, D. Fisher, T. Hunkapiller, D. Pereira, S. M. Weissman, H. Uehara, S. Nathenson, and L. Hood.** 1981. Three cDNA clones encoding mouse transplantation antigens: homology to immunoglobulin genes. Cell **24:**125-34.

262. **Stern-Ginossar, N., N. Elefant, A. Zimmermann, D. G. Wolf, N. Saleh, M. Biton, E. Horwitz, Z. Prokocimer, M. Prichard, G. Hahn, D. Goldman-Wohl, C. Greenfield, S. Yagel, H. Hengel, Y. Altuvia, H. Margalit, and O. Mandelboim.** 2007. Host immune system gene targeting by a viral miRNA. Science **317:**376-81.

263. **Stevens, D. P., L. F. Barker, A. S. Ketcham, and H. M. Meyer, Jr.** 1970. Asymptomatic cytomegalovirus infection following blood transfusion in tumor surgery. Jama **211:**1341-4.

264. **Stingley, S. W., J. J. Ramirez, S. A. Aguilar, K. Simmen, R. M. Sandri-Goldin, P. Ghazal, and E. K. Wagner.** 2000. Global analysis of herpes simplex virus type 1 transcription using an oligonucleotide-based DNA microarray. J Virol **74:**9916-27.

265. **Streblow, D. N., Dumortier, J., Moses, A.V., Orloff, S.L., and Nelson, J.A.** 2008. Mechanisms of Cytomegalovirus-Accelerated Vascular Disease: Induction of Paracrine Factors That Promote Angiogenesis and Wound Healing, p. 397-415. *In* T. E. a. S. Shenk, M.F. (ed.), Human Cytomegaloviruses. Springer, Verlag Berlin Heidelberg.

266. **Streblow, D. N., and J. A. Nelson.** 2003. Models of HCMV latency and reactivation. Trends Microbiol **11**:293-5.

267. **Streblow, D. N., C. Söderberg-Nauclér, J. Vieira, P. Smith, E. Wakabayashi, F. Rutchi, K. Mattison, Y. Altschuler, and J. A. Nelson.** 1999. The human cytomegalovirus chemokine receptor US28 mediates vascular smooth muscle cell migration. Cell **99**:511-20.

268. **Streblow, D. N., K. W. van Cleef, C. N. Kreklywich, C. Meyer, P. Smith, V. Defilippis, F. Grey, K. Fruh, R. Searles, C. Bruggeman, C. Vink, J. A. Nelson, and S. L. Orloff.** 2007. Rat Cytomegalovirus Gene Expression In Cardiac Allograft Recipients Is Tissue Specific And Does Not Parallel The Profiles Detected In Vitro. J Virol.

269. **Streblow, D. N., K. W. van Cleef, C. N. Kreklywich, C. Meyer, P. Smith, V. Defilippis, F. Grey, K. Fruh, R. Searles, C. Bruggeman, C. Vink, J. A. Nelson, and S. L. Orloff.** 2007. Rat cytomegalovirus gene expression in cardiac allograft recipients is tissue specific and does not parallel the profiles detected in vitro. J Virol **81**:3816-26.

270. **Suss, G., and K. Shortman.** 1996. A subclass of dendritic cells kills CD4 T cells via Fas/Fas-ligand-induced apoptosis. J Exp Med **183**:1789-96.

271. **Swack, N. S., and G. D. Hsiung.** 1982. Natural and experimental simian cytomegalovirus infections at a primate center. J Med Primatol **11**:169-77.

272. **Sylwester, A. W., B. L. Mitchell, J. B. Edgar, C. Taormina, C. Pelte, F. Ruchti, P. R. Sleath, K. H. Grabstein, N. A. Hosken, F. Kern, J. A. Nelson, and L. J. Picker.** 2005. Broadly targeted human

cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory compartments of exposed subjects. J Exp Med **202:**673-85.

273. **Szabolcs, P., D. Avigan, S. Gezelter, D. H. Ciocon, M. A. Moore, R. M. Steinman, and J. W. Young.** 1996. Dendritic cells and macrophages can mature independently from a human bone marrow-derived, post-colony-forming unit intermediate. Blood **87:**4520-30.

274. **Tabeta, K., P. Georgel, E. Janssen, X. Du, K. Hoebe, K. Crozat, S. Mudd, L. Shamel, S. Sovath, J. Goode, L. Alexopoulou, R. A. Flavell, and B. Beutler.** 2004. Toll-like receptors 9 and 3 as essential components of innate immune defense against mouse cytomegalovirus infection. Proc Natl Acad Sci U S A **101:**3516-21.

275. **Taylor-Wiedeman, J., P. Sissons, and J. Sinclair.** 1994. Induction of endogenous human cytomegalovirus gene expression after differentiation of monocytes from healthy carriers. J Virol **68:**1597-604.

276. **Taylor, R. T., and W. A. Bresnahan.** 2005. Human cytomegalovirus immediate-early 2 gene expression blocks virus-induced beta interferon production. J Virol **79:**3873-7.

277. **Thale, R., P. Lucin, K. Schneider, M. Eggers, and U. H. Koszinowski.** 1994. Identification and expression of a murine cytomegalovirus early gene coding for an Fc receptor. J Virol **68:**7757-65.

278. **Thibodeau, J., M. C. Bourgeois-Daigneault, G. Huppe, J. Tremblay, A. Aumont, M. Houde, E. Bartee, A. Brunet, M. E. Gauvreau, A. de Gassart, E. Gatti, M. Baril, M. Cloutier, S. Bontron, K. Fruh, D.**

Lamarre, and V. Steimle. 2008. Interleukin-10-induced MARCH1 mediates intracellular sequestration of MHC class II in monocytes. Eur J Immunol **38:**1225-30.

279. **Tomasec, P., V. M. Braud, C. Rickards, M. B. Powell, P. McSharry, S. Gadola, V. Cerundolo, L. K. Borysiewicz, A. McMichael, and G. W. G. Wilkinson.** 2000. Surface expression of HLA-E, an Inhibotor of natural killer cells, enhanced by human cytomegalovirus gpUL40. Science **287:**1031-33.

280. **Tomasec, P., E. C. Wang, A. J. Davison, B. Vojtesek, M. Armstrong, C. Griffin, B. P. McSharry, R. J. Morris, S. Llewellyn-Lacey, C. Rickards, A. Nomoto, C. Sinzger, and G. W. Wilkinson.** 2005. Downregulation of natural killer cell-activating ligand CD155 by human cytomegalovirus UL141. Nat Immunol **6:**181-8.

281. **Tomazin, R., J. Boname, N. R. Hegde, D. M. Lewinsohn, Y. Altschuler, T. R. Jones, P. Cresswell, J. A. Nelson, S. R. Riddell, and D. C. Johnson.** 1999. Cytomegalovirus US2 destroys two components of the MHC class II pathway, preventing recognition by CD4+ T cells. Nat Med **5:**1039-43.

282. **Tomazin, R., J. Boname, N. R. Hegde, D. M. Lewinsohn, Y. Altschuler, T. R. Jones, P. Cresswell, J. A. Nelson, S. R. Ridell, and D. C. Johnson.** 1999. Cytomegalovirus US2 destroys two compartment of the MHC class II pathway, preventing recognition by CD4+t cells. Nature medicine **5:**1039-43.

283. **Torok-Storb, B., P. Simmons, D. Khaira, D. Stachel, and D. Myerson.** 1992. Cytomegalovirus and marrow function. Ann Hematol **64 Suppl:**A128-31.

284. **Ulbrecht, M., S. Martinozzi, M. Grzeschik, H. Hengel, J. W. Ellwart, M. Pla, and E. H. Weiss.** 2000. The Human cytomegalovirus UL40 gene product contains a ligand for HLA-E and prevents NK cell mediated Lysis. The Journal of Immunology **164:**5019-22.

285. **Valantine, H. A., S. Z. Gao, G. Santosh, and e. al.** 1999. Impact of prophylactic immediate post-transplant ganciclovir on development of transplant atherosclerosis. A post-hoc analysis of a randomized, placebo-controlled study. Circulation **100:**61-66.

286. **van der Strate, B. W., J. L. Hillebrands, S. S. Lycklama a Nijeholt, L. Beljaars, C. A. Bruggeman, M. J. Van Luyn, J. Rozing, T. H. The, D. K. Meijer, G. Molema, and M. C. Harmsen.** 2003. Dissemination of rat cytomegalovirus through infected granulocytes and monocytes in vitro and in vivo. J Virol **77:**11274-8.

287. **van Leeuwen, E. M., E. B. Remmerswaal, M. H. Heemskerk, I. J. ten Berge, and R. A. van Lier.** 2006. Strong selection of virus-specific cytotoxic CD4+ T-cell clones during primary human cytomegalovirus infection. Blood **108:**3121-7.

288. **van Niel, G., R. Wubbolts, and W. Stoorvogel.** 2008. Endosomal sorting of MHC class II determines antigen presentation by dendritic cells. Curr Opin Cell Biol **20:**437-44.

289. **Varani, S., G. Frascaroli, D. Gibellini, L. Potena, T. Lazzarotto, R. M. Lemoli, C. Magelli, C. Soderberg-Naucler, and M. P. Landini.** 2005. Impaired dendritic cell immunophenotype and function in heart transplant patients undergoing active cytomegalovirus infection. Transplantation **79:**219-27.

290. **Varani, S., G. Frascaroli, M. Homman-Loudiyi, S. Feld, M. P. Landini, and C. Soderberg-Naucler.** 2005. Human cytomegalovirus inhibits the migration of immature dendritic cells by down-regulating cell-surface CCR1 and CCR5. J Leukoc Biol **77:**219-28.

291. **Varnum, S. M., D. N. Streblow, M. E. Monroe, P. Smith, K. J. Auberry, L. Pasa-Tolic, D. Wang, D. G. Camp, 2nd, K. Rodland, S. Wiley, W. Britt, T. Shenk, R. D. Smith, and J. A. Nelson.** 2004. Identification of Proteins in Human Cytomegalovirus (HCMV) Particles: the HCMV Proteome. J Virol **78:**10960-6.

292. **Voigt, V., C. A. Forbes, J. N. Tonkin, M. A. Degli-Esposti, H. R. Smith, W. M. Yokoyama, and A. A. Scalzo.** 2003. Murine cytomegalovirus m157 mutation and variation leads to immune evasion of natural killer cells. Proc Natl Acad Sci U S A **100:**13483-8.

293. **Wagner, M., S. Jonjic, U. H. Koszinowski, and M. Messerle.** 1999. Systematic excision of vector sequences from the BAC-cloned herpesvirus genome during virus reconstitution. J. Virol. **73:**7056-60.

294. **Walseng, E., O. Bakke, and P. A. Roche.** 2008. Major histocompatibility complex class II-peptide complexes internalize using a clathrin- and dynamin-independent endocytosis pathway. J Biol Chem **283:**14717-27.

295. **Wang, D., and T. Shenk.** 2005. Human cytomegalovirus UL131 open reading frame is required for epithelial cell tropism. J Virol **79:**10330-8.

296. **Wang, D., and T. Shenk.** 2005. Human cytomegalovirus virion protein complex required for epithelial and endothelial cell tropism. Proc Natl Acad Sci U S A **102:**18153-8.

297. **Wang, X., D. Y. Huang, S. M. Huong, and E. S. Huang.** 2005. Integrin alphavbeta3 is a coreceptor for human cytomegalovirus. Nat Med **11:**515-21.

298. **Wang, X., S. M. Huong, M. L. Chiu, N. Raab-Traub, and E. S. Huang.** 2003. Epidermal growth factor receptor is a cellular receptor for human cytomegalovirus. Nature **424:**456-61.

299. **Weller, T. H., J. B. Hanshaw, and D. E. Scott.** 1960. Serological differentiation of viruses responsible for cytomegalic inclusion disease. J Virol. **12:**130-32.

300. **Weller, T. H., J. C. Macauley, J. M. Craig, and P. Wirth.** 1957. Isolation of intranuclear inclusion producing agents from infants with illnesses resembling cytomegalic inclusion disease. Proc. Soc. Exp. Biol. Med. **94:**4-12.

301. **Welte, S. A., C. Sinzger, S. Z. Lutz, H. Singh-Jasuja, K. L. Sampaio, U. Eknigk, H. G. Rammensee, and A. Steinle.** 2003. Selective intracellular

retention of virally induced NKG2D ligands by the human cytomegalovirus UL16 glycoprotein. Eur J Immunol **33:**194-203.

302. **Wiertz, E. J., R. Devlin, H. L. Collins, and M. E. Ressing.** 2007. Herpesvirus interference with major histocompatibility complex class II-restricted T-cell activation. J Virol **81:**4389-96.

303. **Wiertz, E. J., T. R. Jones, L. Sun, M. Bogyo, H. J. Geuze, and H. L. Ploegh.** 1996. The human cytomegalovirus US11 gene product dislocates MHC class I heavy chains from the endoplasmic reticulum to the cytosol. Cell **84:**769-79.

304. **Wiertz, E. J., D. Tortorella, M. Bogyo, J. Yu, W. Mothes, T. R. Jones, T. A. Rapoport, and H. L. Ploegh.** 1996. Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction. Nature **384:**432-8.

305. **Wills, M. R., Carmichael, Andrew J., and Sissons, J.G. Patrick.** 2006. Adaptive Cellular Immunity to Human Cytomegalovirus, p. 341-366. *In* M. Reddehase (ed.), Cytomegaloviruses Molecular Biology and Immunology. Caister Academic Press, Norfolk.

306. **Woolf, N. K., F. J. Koehrn, J. P. Harris, and D. D. Richman.** 1989. Congenital cytomegalovirus labyrinthitis and sensorineural hearing loss in guinea pigs. J Infect Dis **160:**929-37.

307. **Wu, J., N. J. Chalupny, T. J. Manley, S. R. Riddell, D. Cosman, and T. Spies.** 2003. Intracellular retention of the MHC class I-related chain B

ligand of NKG2D by the human cytomegalovirus UL16 glycoprotein. J Immunol **170:**4196-200.

308. **Wu, L., and A. Dakic.** 2004. Development of dendritic cell system. Cell Mol Immunol **1:**112-8.

309. **Wu, T. C., R. H. Hruban, R. F. Ambinder, M. Pizzorno, D. E. Cameron, W. A. Baumgartner, B. A. Reitz, G. S. Hayward, and G. M. Hutchins.** 1992. Demonstration of cytomegalovirus nucleic acids in the coronary arteries of transplanted hearts. Am J Pathol **140:**739-47.

310. **Yagyu, K., P. J. van Breda Vriesman, A. M. Duijvestijn, C. A. Bruggeman, and G. Steinhoff.** 1993. Reactivation of cytomegalovirus with acute rejection and cytomegalovirus infection with obliterative bronchiolitis in rat lung allografts. Transplant Proc **25:**1152-4.

311. **Yang, G. X., Z. X. Lian, K. Kikuchi, Y. Moritoki, A. A. Ansari, Y. J. Liu, S. Ikehara, and M. E. Gershwin.** 2005. Plasmacytoid dendritic cells of different origins have distinct characteristics and function: studies of lymphoid progenitors versus myeloid progenitors. J Immunol **175:**7281-7.

312. **Yu, D., M. C. Silva, and T. Shenk.** 2003. Functional map of human cytomegalovirus AD169 defined by global mutational analysis. Proc Natl Acad Sci U S A **100:**12396-401.

313. **Zhu, J., and W. E. Paul.** 2008. CD4 T cells: fates, functions, and faults. Blood **112:**1557-69.

314. **Ziegler, H., W. Muranyi, H. G. Burgert, E. Kremmer, and U. H. Koszinowski.** 2000. The luminal part of the murine cytomegalovirus

glycoprotein gp40 catalyzes the retention of MHC class I molecules. Embo J **19:**870-81.

315. **Ziegler, H., R. Thale, P. Lucin, W. Muranyi, T. Flohr, H. Hengel, H. Farrell, W. Rawlinson, and U. H. Koszinowski.** 1997. A mouse cytomegalovirus glycoprotein retains MHC class I complexes in the ERGIC/cis-Golgi compartments. Immunity **6:**57-66.

316. **Zimmermann, A., M. Trilling, M. Wagner, M. Wilborn, I. Bubic, S. Jonjic, U. Koszinowski, and H. Hengel.** 2005. A cytomegaloviral protein reveals a dual role for STAT2 in IFN-{gamma} signaling and antiviral responses. J Exp Med **201:**1543-53.

317. **Zimmermann, A. a. H., Hartmut.** 2006. Cytomegalovirus Interference with Interferons, p. 321-339. *In* M. J. Reddehase (ed.), Cytomegaloviruses Molecular Biology and Immunology. Caister Academic Press, Norfolk.